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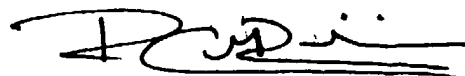
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Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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Mosaic Infectious Bursal Disease Virus vaccines

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Title: Mosaic Infectious Bursal Disease Virus vaccines.

The invention relates to Infectious Bursal Disease
Virus (IBDV) vaccines.

Infectious Bursal Disease (IBD), an infectious disease
among young chickens, was first recognized in 1957 in
5   Gumboro, Delaware, USA and formally documented by
Cosgrove (Cosgrove, 1962; Lasher and Shane, 1994). As a
result, the disease is often referred to as Gumboro. Not
long after IBD was first reported, it was being
recognized in poultry populations throughout the world
10   (Lasher and Shane, 1994). IBD is caused by a virus (IBDV)
classified as a Birnavirus (Dobos et al., 1979). Two
different IBDV serotypes exist: serotype I and II
(Jackwood et al., 1982; McFerran et al., 1980). Isolates
belonging to serotype I are highly pathogenic for
15   chickens. Serotype II isolates, which are mainly
recovered from turkeys, have never been reported to
induce clinical signs in chickens and are regarded as
apathogenic (Ismail et al., 1988). Infectious bursal
disease or Gumboro is a highly contagious disease for
20   young chickens, and is responsible for severe losses in
poultry industries. In birds surviving an acute
infection, lymphoid cells in the bursa of Fabricius are
destroyed, resulting in B-cell dependent
immunodeficiency. This causes increased susceptibility to
25   disease caused by otherwise harmless agents. A central
role in the pathogenesis of Gumboro is played by the
bursa, which is representing the target organ of the
virus.

IBDV infections were initially recognized by whitish
30   or watery diarrhea, anorexia, depression, trembling,
weakness, and death. This clinical IBD was generally seen
in birds between three and eight weeks of age. The course
of the disease runs approximately 10 day in a flock

in commercial poultry than the clinical disease. It is generally seen in birds less than three weeks of age.

5 This early infection results in a B-lymphocyte depletion of the bursa of Fabricius. The bird is immunologically crippled and unable to respond fully to vaccinations or field infections. In susceptible chickens, damage caused by IBDV can be seen within two to three days after

10 exposure to virulent virus. Initially, the bursa swells (3 days post-exposure) with edema and hemorrhages and then begins to show atrophy (7-10 days). IBD virus is especially cytopathic to certain B-lymphocytes. The highest concentration of these specific B-lymphocytes is

15 found in the bursa. Destruction of the B-lymphocytes by IBD field virus may result in an incomplete seeding of these cells in secondary lymphoid tissue. As a result of the depletion of B-lymphocytes, surviving birds are immunocompromised during the remaining of their live

20 time.

IBDV is found worldwide, and IBDV specific antibodies have even been found in Antarctic penguins (Gardner et al., 1997). The prevalence of clinical IBD is relatively low compared to the prevalence of subclinical

25 IBD. IBDV is very resistant to common disinfectants and has been found in lesser mealworms, mites, and mosquitoes. These facts correlate with field experience of reoccurring IBD problems on a farm, despite clean-up efforts. Infection with IBDV results in a strong antibody

30 response against IBD, which is capable of neutralizing this virus. Most likely as a result of vaccination, antigenic variant isolates of serotype I were isolated in the Delaware area (USA). These isolates have been shown to cause bursa atrophy in as little as three days post-

35 infection without inflammation of the bursa. Despite their change in antigenicity these antigenic variants do not form a distinct serotype. After the occurence of

Van den Bos, 1995). These very virulent field isolates
were capable of establishing themselves in the face of
5   high levels of maternal antibodies which normally were
protective. These vvIBDV cause more severe clinical signs
during an outbreak and are now found globally (e.g.
Europe, Japan, Israel and Asia).

IBDV belongs to the family of Birna viruses which
10   include Infectious Bursal Disease Virus (IBDV) isolated
from chickens, Infectious Pancreatic Necrosis Virus
(IPNV) isolated from Fish, Drosophila X Virus (DXV)
isolated from fruit fly, and Tellina virus (TV) and
Oyster Virus (OV) both isolated from bivalve molluscs
15   (Dobos et al., 1979). Birna viruses have a dsRNA genome
which is divided over two genome segments (the A- and B-
segment). The A-segment (3.3 kbp) contains two partly
overlapping open reading frames (ORFs). The first,
smallest ORF encodes the non-structural Viral Protein 5
20   (VP5, 17 kDa). The second ORF encodes a polyprotein (1012
amino acid, 110 kDa), which is autocatalytically cleaved.
The exact position of these cleavage sites is unknown.
From SDS-Page analysis of *in vitro* translated IBDV RNA it
is known that the polyprotein is rapidly cleaved into
25   three proteins: pVP2 (48 kDa), VP4 (29 kDa) and VP3 (33
kDa). During *in vivo* virus maturation pVP2 is processed
into VP2 (38 kDa), probably resulting form site-specific
cleavage of the pVP2 by a host cell encoded protease
(Kibenge et al., 1997). VP2 and VP3 are the two proteins
30   that constitute the single shell of the virion. The B-
segment (2.9 kbp) contains one large ORF, encoding the 91
kDa VP1 protein. This protein contains a consensus RNA
dependent RNA polymerase motive (Bruenn, 1991).
Furthermore, this protein has been reported to be linked
35   to the 5'-ends of the genomic RNA segments (Viral Protein
genome-linked, VPg). The nucleotide sequence of internal

1995) have determined the 5'- and 3'-termini of several IBDV isolates (CU-1, CU-1M, P-2 and 23/82), and by combining the internal and terminal sequences, Mundt and Muller established the complete nucleotide sequence of a serotype I A-segment (3261 bp) and B-segment (2827 bp). This provided the way to generate an infectious (recombinant) copy (rIBDV) of IBDV serotype I, by knowing the complete sequence dsRNA sequence of IBDV genome and by using one of several methods to generate infectious copy virus (see for example Boyer et al, Virology 198:415-426, 1994), Mundt and Vakharia indeed produced infectious rIBDV serotype I from cDNA (Mundt and Vakharia, 1996). Full length cDNA of a serotype I IBDV, preceeded by a T7 promoter, was thereby used as a template for T7 RNA polymerase using a method described by Weiland and Dreher (Weiland and Dreher, 1989). The *in vitro* generated mRNA, containing a cap-structure at its 5'-end, was subsequently transfected into eukaryotic cells (VERO cells) using a liposome formulation (Lipofectin, GibcoBRL). The supernatant of the transfected cells contained infectious rIBDV after incubation during 36h in the CO_2 incubator at 37° C (Mundt and Vakharia, 1996; (WO 98/09646)). In addition, Lim et al. introduced two amino acid mutations (D279N and A284T) into the cDNA of vvIBDV isolate HK46 (Lim et al., 1999). These mutations were most probably based on data of Yamaguchi et al. (Yamaguchi et al., 1996), which showed that these specific mutations were found in two independent experiments in which very virulent IBDV isolates lost their very virulent charcater by adaptation and growth on primary CEF cells. Lim et al obtained a rIBDV isolate which possessed the phenotype of a CEF-culture adapted isolate, i.e. a rIBDV isolate which can be propagated, i.e. is able to infect, multiply and be

al., 1999). Furthermore, although cDNA of IBDV can be used to produce infectious IBDV, the exact mechanism of
5   replication has not been elucidated yet. Data exist which are in support of a semi-conservative genome replication model for Birnaviradae (Bernard, 1980; Mertens et al., 1982).

Now and then IBDV variants are detected in the field
10   or are created in cell-culture in the laboratory (Muller, 1987) that are genetic re-assortments of serotype I and II strains of IBDV, in that they contain one genomic segment derived from the one serotype, and another segment derived from the other serotype. Such segment re-
15   assorted (srIBDV) strains (also called chimeric IBDV) not only occur in nature, but have recently been generated from cDNA as a well, by Vahkaria and Mundt (WO 98/09646). Vaccination using attenuated field isolates worked sufficiently well until antigenic derivatives were found
20   in the Delaware region of the USA starting in 1985 (isolates Del A, D, G and E) (Snyder, 1990). These field isolates were missing an important virus neutralizing epitope. The change of this epitope is characterized by the lack of binding of the virus neutralizing monoclonal
25   antibody (Mab) B69 (Snyder et al., 1988a). The antibodies induced by vaccination with classical IBDV vaccines appeared to be less protective against these antigenic IBDV variants. Inactivated vaccines based upon antigenic IBDV variants were subsequently produced and were found
30   to protect effectively against these antigenic variants of IBDV. After the Delaware variant a second antigenic variant IBDV was isolated. This variant was recovered from the Delmarva region (USA) and was referred to as the GLS variant. The GLS variant is characterized by the
35   absence of epitopes for both the virus neutralizing Mab B69 and R63 (Snyder et al., 1988b). After identifying these antigenic variants, a large survey was performed

BK44, in addition to those for Mabs B69 and R63 (Snyder, 1990). No further reports of antigenic variants have been published in the USA or in other parts of the world. Whether this is due to non-existence of new variant IBDV isolates or whether new antigenic variants just have not been detected due to the lack of extensive surveys or the lack of discriminating monoclonal antibodies is unclear.

The nucleotide sequence of the polyprotein encoding part of the A-segment of the Del, the GLS and the DS326 antigenic variant IBDV isolates has been determined (Vakharia et al., 1994). Most of the amino acid changes were found in a specific region of the VP2 protein, the so-called hypervariable region. Furthermore it was found that the epitopes which are capable of inducing neutralizing antibodies are conformation dependent and are clustered in the hypervariable region. This region consists of a domain with a high hydrophobicity index (amino acid 224 to 314 of pVP2, corresponding with amino acid 224 to 314 of the polyprotein) which is flanked by two small hydrophilic regions, each spanning about 14 amino acids (Vakharia et al., 1994; Heine et al., 1991). Amino acid substitution both within the hydrophobic region and within the hydrophilic regions might be involved in the antigenic variant character of these isolates.

After the problems caused by the antigenic variant IBDV isolates in the USA, the poultry industry in Europe was affected by very virulent IBDV (vvIBDV) isolates (Berg et al., 1991; Chettle et al., 1989). The vvIBDV isolates cause more severe clinical signs during an outbreak and are able to break through levels of antibodies which are protective against classical IBDV isolates. The molecular determinants which distinguish vvIBDV from classical IBDV isolates are not exactly known. It is known however, that the pathogenicity of

phenotype is likely to be due to the change in target
cell tropism of the adapted virus. This change in cell
tropism may be due to the loss of bursa cell receptor
binding capability of the cell culture adapted very
virulent IBDV isolate. Another possibility is that the
cell culture adapted very virulent IBDV isolate is able
to infect non-bursa cells, resulting in large reduction
of IBDV load in the primary target cells (bursa cells).
Form the published results (Yamaguchi et al., 1996), it
is clear that a recombinant IBDV (rIBDV) which is based
upon the cDNA of a cell culture adapted very virulent
isolate will never yield a vaccine which meets the
demands of being able to break through high levels of
maternal antibodies and induce a high enough immune
response.

No specific antibodies, that exclusively recognize
the vvIBDV isolates have been described yet (Eterradossi
et al., 1997)). The lack of discriminating antibodies
makes direct diagnosis difficult. Most attention has been
given to sequence comparison between the hypervariable
region of VP2 of classical isolates and of very virulent
isolates. Sequence analysis of the vvIBDV isolate UK661
showed that only three unique (i.e. not found in non-
vvIBDV isolates) amino acid substitution are present
within the hypervariable region of the VP2 protein. One
amino acid substitution is present within the remaining
part of the pVP2 protein, while 5 unique amino acid
mutations are present within the VP4 encoding part of the
polyprotein and 6 in the VP3 encoding part. (Brown and
Skinner, 1996). The smaller ORF of the UK661 isolate A-
segment, encoding the VP5 protein, contains 2 unique
amino acid substitutions. Additionally 16 unique amino
acid substitutions were found in the VP1 protein encoded
by the B-segment of this vvIBDV isolate. The virulent
phenotype of the vvIBDV might be of the

with the classical or antigenic variant isolates. Serial
passage on embryonated eggs of a vvIBDV isolate (OKYM)
5   resulted in the appearance of a derivative isolate
(OKYMT) which is able to grow on Chicken Embryo
Fibroblast (CEF) cells and has lost its virulence. This
adaptation was reported to be the result of 7 nucleotide
substitutions in the polyprotein encoding part of the
10   genome. Whether additional nucleotide substitutions (or
deletions) were present in remaining parts of the A- or
B-segment (e.g. untranslated regions, VP1 encoding
region, and VP5 encoding region) was not determined
(Yamaguchi et al., 1996). The reported nucleotide
15   substitutions result in 5 amino acid substitutions. Three
of these amino acid substitutions were located in the
hydrophobic part of the hypervariable region (I256T,
D279N, A284T) of VP2, one in the hydrophilic part located
downstream of the hypervariable region (S315F) of VP2,
20   and one in VP3 (A805T) (Yamaguchi et al., 1996). In an
independent experiment, Yamaguchi et al. found that the
adaptation of vvIBDV isolate TKSM into TKSMT resulted
also in the A284T and D279N substitutions. The A284T
substitution correlated in their analysis completely with
25   adaptation onto CEF cells and loss of virulence. The
D279N substitution was also present in both CEF-adapted
vvIBDV isolates (OKYMT and TKSMT) and is potentially
also important for growth on CEF cells and loss of
virulence. The non-CEF adapted, classical IBDV isolate
30   GBF-1 has on the other hand an asparagine at position
279, in combination with alanine at position 284 and
cannot grow on CEF cells, so the single substitution
D279N does not account loss of virulence and growth on
CEF cells. The amino acid changes in the VP2 apparently

is was shown that amino acid substitution, A284T in combination with D279N is indeed enough to turn a non-CEF-adapted very virulent IBDV isolate into a CEF-adapted isolate. Lim et al. introduced these two amino acid substitutions into the A-segment cDNA of vvIBDV isolate HK46 (Lim et al., 1999). After transfection of this cDNA, Lim et al obtained a rIBDV isolate which possessed the phenotype of a CEF-culture adapted isolate, i.e. a rIBDV isolate which is able to infect and multiply in CEF cells. The virulence of this rIBDV isolate was not assessed in chickens. Note worthy, Lim et al. were unable to produce a recombinant infectious vvIBDV isolate using the unmodified cDNA of the HK46 isolate (Lim et al., 1999).

The goal of vaccination against IBD is prevention of subclinical and clinical IBD and the economic aspects of each. Effective vaccination for IBD can be divided into the following categories:

Protection of the developing bursa in broilers, breeders and layers.

Prevention of clinical disease in broilers, breeders and layers.

Priming and boosting of breeders.

To minimize the immunosuppressive effects of IBDV, the young chick must be protected. Protection of the very young can be achieved through high enough levels of maternal antibodies passed from the breeder hen to her progeny. Vaccination of the very young chick itself may not be successful since onset of protection after vaccination is between three and five days. When a bird, lacking maternal antibodies against IBDV, is exposed to a pathogenic IBDV field strain, damage will occur within 24-48 hours.

vaccination is not considered to be adequate. Boosting is
the term commonly associated with the administration of a
5   final IBDV vaccination prior to the onset of lay. This is
done to increase the circulating antibody in the hen and
hence the maternal antibodies in the progeny. Both
inactivated (oil emulsion vaccine) and live vaccines
(IBDV) have been used for this purpose. The use of a live
10   vaccine in an older bird will result in an increase of
antibodies; however, large variations in antibody titers
are often seen. These variations result in progeny
becoming susceptible to field challenge from as early as
a few days after hatching to 21 days after hatching. The
15   use of inactivated IBDV vaccines gives a higher antibody
titer as well as a decrease of variation between antibody
titers of birds belonging to the same flock. The levels
of maternal antibodies necessary to neutralize IBD vary
with the invasiveness and pathogenicity of the field
20   strain. In practical terms, if a very virulent IBDV
isolate is present, higher maternal antibody levels are
desired (see Table 1 for an overview of virulence of
field isolates and strength of vaccines). Yet, for
effective vaccination, avoiding interference with
25   maternal antibodies is needed to induce a good immune
response. Clinical IBD is typically seen between three
and six weeks of age. The immune response of the chick
must be stimulated as the passive protection is
declining. The timing of the active vaccination may be
30   estimated by the breeder or chick titer and the half-life
of antibodies of approximately 3.5 days (De Wit and Van
Loon, 1998; Kouwenhoven and Van den Bos, 1995). The
levels of maternal antibodies tend to vary within a
population. This variation might be a result of variation
35   in the antibodies levels of the breeder hen. Also the
mixing of progeny from several breeder flocks (e.g.

variation (CV) in mean maternal antibody titers is too wide, it may be recommended to vaccinate twice (with a 10-day interval) or to vaccinate early with a hot vaccine (in the presence of a high antigenic pressure).

The average titer of antibody against IBDV in a flock will decline in time (Fig. 1). As a result of the decrease in average antibody titers, an immunity gap will occur. The best results are obtained if the immunity gap is as short as possible and is as early as possible, with a minimum of 2 weeks after hatching. There should be at least sufficient immunity after active vaccination at the age of 4 weeks, since many handlings occur in the houses from that time point with risks of introducing field virus. Therefore farmers like to vaccinate at 2 weeks or even before. Intermediate vaccines are often unable to break through the average IBDV antibody titer of the broiler at two weeks after hatching (Fig. 1). If there is a high variation in mean maternal antibody titers, some chicks will be effectively vaccinated with intermediate vaccines, others not. To circumvent those problems, hot vaccines are being used. A drawback of usage of hot vaccines is that the bursa of chickens with low to moderate maternal antibody titers will be (partly) damaged.

There is a wide variety of IBDV vaccines available. Important aspects in vaccination strategies are the ability of the virus to replicate in the face of maternal antibody (invasiveness of the vaccine) and the spectrum of antigenic content (including antigenic variants). The ability of a vaccine virus to replicate in the face of maternal antibodies allows live vaccines to be categorized into three main groups: mild, intermediate, and intermediate plus or hot vaccines (see Table 1). The initial vaccines for IBD were derived from classical

maternal antibodies, these vaccines could cause extensive bursal atrophy resulting in immunosuppression. Mild

5 vaccines were subsequently developed to be used in these young birds. The attenuation of classical IBDV was done in tissue culture systems. Traditionally, attenuated strains for vaccines are generated by adapting IBDV strains to chicken embryoblast (CEF) cells or other

10 appropriate cells or cell lines through serial passages. These vaccines are not immunosuppressive even when used in birds having no maternal antibodies. However, moderate and high levels of antibodies easily neutralize them. As breeder programs developed (including the use of

15 adjuvant, inactivated vaccines), higher levels of maternal antibodies were generated in progeny. This reduces the effectiveness of these mild vaccines.

Intermediate strength vaccines were to overcome the inadequacies of the mild vaccines. Some of the

20 intermediate vaccines were developed by cloning a field isolate on chicken cell cultures. Intermediate strength vaccines are capable of establishing immunity in birds with moderate levels of maternal antibodies. These vaccines will cause some bursal atrophy in birds without

25 maternal antibodies, but are considered not immunosuppressive.

Hot (strong) or intermediate plus vaccines were developed after the first outbreaks with vvIBDV. These vvIBDV isolates could break through higher levels of

30 maternal immunity than the vaccines that were on the market at that time. Vaccination with intermediate vaccines came always too late in situations with high infection pressure with vvIBDV. Hot vaccines consist of vvIBDV strains with low to moderate passage in

35 embryonated eggs or bursa derived IBDV of chickens infected with vvIBDV isolates. Adapting vvIBDV on cells

vaccine. Hot or intermediate plus vaccines are desirably able to circumvent maternal immunity at an earlier age than intermediate vaccines but spread more within a flock. If intermediate plus and hot vaccines are used in chickens with moderate to high levels of maternal antibodies, there is no negative side effect on the bursa (Kouwenhoven and Van den Bos, 1995). If these vaccines are used in chickens with low to moderate levels of maternal immunity, this causes depletion of lymphoid cells in the bursa and a severe depletion of peripheral blood-B cells is found (Ducatelle et al., 1995). Although a recovery of bursal function has been observed, these vaccines should be used with precautions.

Live vaccines must be given in a way in which the virus will preferably reach the bursa where it will quickly multiply and induce an immune response. Possible routes for application of live vaccines include drinking water, spray, subcutaneous and in ovo. Inactivated IBD vaccines are used in broiler breeders. They differ in some of the same ways as live vaccines. Their efficacy depends upon the spectrum of antigens they contain. Injectable oil-emulsion products may be given subcutaneously or intramuscularly.

A continuous monitoring of the field situation using an integrated quality control scheme including serology, can be a valuable tool for continuously adapting preventive vaccination programs to changing epidemiological conditions. Also a continuous follow-up of the epidemiological situation will allow to anticipate the development of major epidemics (Ducatelle et al., 1995). However, the ability of diagnostic laboratories to monitor IBD with meaningful definitive data is difficult. Serology is important but can be confusing when all birds monitored from commercial broiler flocks have high levels
of the same spectrum of circulating antibodies

induced by IBDV field infections. If it were possible to discriminate between IBDV antibody response to field

5 virus and IBDV vaccination it is possible to have 'early warning' systems and to start IBDV eradication programs if desired. Only when there is a known difference between the antibody response to the used IBDV vaccine and IBDV field isolates, defined conclusion about whether

10 (sub)clinical signs of IBDV are the result of live IBDV vaccination or of IBDV field isolates can be made.

The invention provides infectious recombinant Infectious Bursal Disease Virus (rIBDV) essentially

15 incapable of growing in a cell that is not derived from a bursa cell or another cell comprising a wild-type IBDV receptor (a non-bursa-cell). A bursa is lymphoid organ, mostly comprising cells that are related to the immune system. In particular, it comprises lymphocytes or

20 lymphocyte precursor cells of sometimes the T-cell- but mainly the B-cell-type, and cells derived thereof, in close relation with monocytes or monocyte derived cells such as macrophages, and also with follicular dendritic cells and antigen presenting cells. In particular, the

25 invention provides rIBDV that is essentially incapable of growing in a cell not listed among above bursa cells or cells derived thereof, such as dentritic cells, monocytes, lymphocytes or cells derived thereof. Herewith the invention provides an rIBDV having retained an

30 important characteristic, in that, an comparison with commonly attenuated IBDV strains, it can not or only little grow in non-bursa cells, such as the well known CEF, QM5 or VERO cells, or other cells that are commonly used for propagating attenuated strains of IBDV. In

35 particular, the invention provides an rIBDV essentially incapable of growing in a non-B-cell derived cell.

previous rIBDV isolates known grow in non-bursa-cell derived cells such as CEF cells (WO98/09646; Lim et al.,

5   1999), thereby for example having lost those very virulent characteristics essential for maintaining in a vaccine strain designed to face above identified problems.

In a preferred embodiment the invention provides in

10   infectious rIBDV having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus (vvIBDV) needed to provide protection against vvIBDV. In particular, vvIBDV is provided that is essentially incapable of growing in a

15   non-bursa-cell derived cell. In particular, as for example demonstrated in the detailed description, the invention provides an rIBDV essentially incapable of growing in a CEF cell, a VERO cell or a QM5 cell, except of course in those CEF, VERO, QM5, or related cells

20   having been provided with the necessary means (such as transgenic receptor or replication system derived from a bursa-cell) needed for replication of classical or very virulent IBDV.

Furthermore, the invention provides an rIBDV wherein

25   the amino acid sequence of protein VP2 comprises no asparagine at amino acid position 279, but for example comprises an amino acid particular for a strain with a very virulent character, such as with aspartic acid at amino acid position 279. Such rIBDV strains as provided

30   by the invention have retained at least part of the very virulent characteristics of vvIBDV, as well as an rIBDV according to the invention wherein the amino acid sequence of protein VP2 comprises no threonine at amino acid position 284, but for example comprises an amino

35   acid particular for a strain with a very virulent character, such as with alanine at amino acid position
284.

from about position 229 to 314, most preferably from about position 214 to 328 as found in a vvIBDV isolate such as shown in Table 8.

The invention furthermore provides a method for obtaining an infectious recombinant copy Infectious Bursal Disease Virus (rIBDV) essentially incapable of growing in a non-bursa-cell derived cell or having at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus (vvIBDV) comprising transfecting at least one first cell with a nucleic acid such as a cDNA or RNA comprising an IBDV genome at least partly derived from a vvIBDV, incubating said first cell in a culture medium, recovering rIBDV from said transfected first cell or said culture medium, propagating said recovered rIBDV in at least one second cell which is permissive for said vvIBDV. A vaccine derived of the recombinant virus as described is also part of this invention. Also a vaccine comprising a chemically or physically inactivated recombinant virus or parts thereof is part of this invention.

Also the attenuated derivatives of initially produced recombinant very virulent IBDV are part of this invention. Such a virus can be attenuated by known methods including serial passage, removing specific nucleic acid sequences, or by site directed mutagenesis. Physiologically acceptable carriers for vaccines of poultry are known in the art and need not be further described herein. Other additives, such as adjuvants and stabilizers, among others, may also be contained in the vaccine in amounts known in the art. Preferably, adjuvants such as aluminum hydroxide, aluminum phosphate, plant and animal oils, and the like, are administered with the vaccine in amounts sufficient to enhance the

glucose; proteins such as albumin or casein; and buffers such as alkaline metal phosphate and the like. A stabilizer is particularly advantageous when a dry vaccine preparation is prepared by lyophilization. The vaccine can be administered by any suitable known method of inoculating poultry including nasally, ophthalmically, by injection, in drinking water, in the feed, by exposure, and the like. Preferably, the vaccine is administered by mass administration techniques such as *in ovo* vaccination, by placing the vaccine in drinking water or by spraying the animals' environment. When administered by injection, the vaccines are preferably administered parenterally. The vaccine of the present invention is administered to poultry to prevent IBD anytime before or after hatching. Poultry is defined to include but not be limited to chickens, roosters, hens, broilers, roasters, breeders, layers, turkeys and ducks. Examples of pharmaceutically acceptable carriers are diluents and inert pharmaceutical carriers known in the art. Preferably, the carrier or diluent is one compatible with the administration of the vaccine by mass administration techniques. However, the carrier or diluent may also be compatible with other administration methods such as injection, eye drops, nose drops, and the like.

As explained above, there is need for an IBDV vaccine that can protect against field infections with IBDV, and preferably against very virulent IBDV (vvIBDV). It is clear that vaccines derived from attenuated classical strains and not from very virulent strains will not be able to sufficiently protect. However, as explained above, simply by adapting and cultivating a vvIBDV strain on a cell or cell-line, such as VERO, CEF

that has at least partly maintained the very virulent or hot character, in order to provide sufficient protection, however, paradoxically, such a desirable vaccine strain would most likely not be able to be sufficiently or substantially be propagated on appropriate cells, such as non-bursa-cell derived VERO, CEF or QM5, deemed needed to obtain said vaccine. In a preferred embodiment, the invention provides a method wherein said first cell is a non-bursa-cell derived cell non-permissive for said vvIBDV, preferably wherein said first cell has additionally been provided with a helpervirus or a viral protein (herein T7-polymerase is used) derived thereof. With the help of such a cell comprising a properly selected helpervirus, e.g. expressing distinct IBDV or Birna virus viral proteins, or of a cell expressing said IBDV or Birna virus viral proteins, (also called a complementary cell) also now defective or deficient rIBDV can be made.

The invention therewith also provides a method to generate infectious Infectious Bursal Disease Virus, by combining cDNA sequences derived from very virulent IBDV (vvIBDV) isolates with cDNA sequences derived from either serotype I classical attenuated IBDV isolates, serotype I antigenic variants of IBDV, or serotype II IBDV isolates, wherein said infectious copy recombinant Infectious Bursal Disease Virus having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus has at least retained the incapacity to substantially be propagated on a vvIBDV non-permissive cell such as a QM5 or CEF cell.

Preferably, a method as provided by the invention provides a vaccine comprising an IBDV genome wherein parts of segments A and/or B derived from a vvIBDV are used combined with parts of segments A and/or B derived from an attenuated IBDV, such as attenuated serotype I or

on susceptible cells. The method provided herein provides a method to generate vvIBDV from the cloned, full length cDNA of a vvIBDV isolate (see Table 5 and 6). After transfection of QM5 cells with cDNA of vvIBDV it is essential that propagation of the generated vvIBDV virus takes place on cells which are permissive for vvIBDV. These permissive cells can for example be found among Bursa-cell derived cells such as primary bursa cells, in chicken in embryo cells, chicken embryo's, or young chickens. Using the method described herein we have for example produced recombinant D6948 (rD6948) using the cDNA derived from the very virulent D6948 IBDV isolate. This rD6948 isolate has the same virulence as the parental D6948 isolate (Table 6).

Preferably, the invention provides a method wherein a permissive second cell is a primary bursa cell, thereby allowing initial propagation of the desired vaccine virus. As explained above, there is a need for a vaccine capable of breaking through maternal immunity of young chickens at an early stage. A desired vaccine should preferably be able to induce a high level of protection in vaccinated young chickens, and should therefore be as immunogenic as very virulent viruses or be almost as immunogenic.

The invention furthermore provides a method to engineer recombinant mosaic IBDV (mIBDV) vaccine which has one or more of the desired phenotypes, i.e. i) being able to break through high levels of maternal antibodies in young chickens and being highly immunogenic, ii) having a reduced pathogenicity compared to wild type very virulent IBDV isolates. In one embodiment, the invention provides an infectious mosaic IBDV (mIBDV) comprising a rIBDV wherein at least one genome segment comprises nucleic acid derived from at least two different Birna virus isolates, when preferred wherein at least one of

vvIBDV permissive cell such as a primary bursa cell. For example, the invention provides mIBDV which consists partly of the genome derived from a classical attenuated isolate (such as CEF94) and partly derived from the genome of a vvIBDV isolate (such as D6948). A recombinant mosaic IBDV (mIBDV), made on the basis of infectious cDNA derived from a very virulent IBDV isolate (D6948), and combined with defined parts of cDNA derived from a cell culture adapted, serotype I, classical IBDV isolate (CEF94) results in a mIBDV isolate which has a reduced pathogenicity compared to wild-type vvIBDV isolates.

Furthermore specific nucleotide substitutions which either do or do not lead to amino acid mutations, or deletion of specific parts of the IBDV genome again leads to an altered phenotype of the generated mIBDV. For example, the replacement of the pVP2 coding region of CEF-94 cDNA with the corresponding region of cDNA of D6948 yielded plasmid pHB36-vvVP2. This plasmid was subsequently transfected into FPT7 (Britton et al., 1996) infected QM5 cells in combination with pHB-34Z. Supernatant of these transfected QM5 cells was subsequently transferred to fresh QM5 cells. None of these QM5 cells reacted positively in an IPMA using specific antibodies for the VP3 protein of IBDV. On the other hand, primary bursa cells, after being overlaid with supernatant of the transfected cells, reacted positively in the same IPMA. The functional feature of being able to enter permissive cells such as QM5 cells is apparently located in the pVP2 coding region of the A-segment. This invention provides a method to generate recombinant vvIBDV (such as rD6948) having a pVP2 sequence exactly as found in a wild-type vvIBDV (here D6948). All very virulent isolates of which the pVP2 sequences has been described thus far have an alanine at position 284 and cannot or only little be propagated on

of a very virulent phenotype (see Table 7 and 8). Herein
we describe a method to generate infectious recombinant
IBDV (rIBDV) having the nucleotide sequence of a wild-
type very virulent IBDV isolate, including the alanine
codon for amino acid 284, and being unable to be
propagated on CEF cells. Furthermore, in our rD6948
isolate we have an aspartic acid present at position 279
in stead of a asparagine commonly found for avirulent
IBDV isolates which can be propagated on CEF cells (Table
7 and 8). The rD6948 is truly a very virulent rIBDV, as
it is unable to grow on CEF cells (Tabel 5), and induces
similar clinical signs and mortality as wild-type very
virulent D6948 isolate (Table 6). Although mIBDV isolate
(mCEF94-vvVP2) did, in contrast to the D6948, rD6948 and
srIBDV-DACB isolates (also having a functional VP2
protein derived from vvIBDV, see Table 6), not cause any
mortality in a 9-days course or body weight loss, it
caused the same reduction in bursa weight after 9 days
post-infection as the wild-type very virulent D6948
isolate.

In yet another embodiment, the invention provided a
mosaic IBDV according to the invention wherein at least
one of said isolates is a serotype II IBDV. Such a mIBDV,
preferably lacking at least one immunodominant epitope
specific for a serotype I IBDV as well is a (r)D6948
derived vaccine virus such as mD6948-s2VP3C1, also having
a functional VP2 protein derived from vvIBDV, allowing
vaccination with a marker vaccine. Vaccination with a
IBDV marker vaccine and subsequent testing with a
corresponding diagnostic test enables the discrimination
between antibodies induced by the vaccine and by
infection with IBDV field isolates. This mIBDV can be
differentiated from all other known wild-type IBDV
isolates, either belonging to serotype I or serotype II,

differentiated from the serological response induced by

IBDV field strains. The marker vaccine provided by the

5   invention, lacking at least one immunodominant epitope,
preferably a serotype I epitope, enables the
discrimination between vaccinated and infected animals by
means of a diagnostic serologic test. Such a mIBDV marker
vaccine is preferably based upon vvIBDV and contains

10   specific sequences originating from classical serotype I
or serotype II IBDV. Such a mIBDV marker vaccine has one
or more of the following characteristics: i) It induces a
protective immune response against vvIBDV field viruses
despite high levels of maternal antibodies. ii) It has a

15   reduced pathogenicity compared to vaccines based upon
wild-type vvIBDV. iii) It for example misses at least one
serotype I specific antigen which enables the serological
discrimination of the mIBDV marker vaccine from all
serotype I IBDV isolates.

20   Also the invention provides a method to produce or
generate tailor made vaccines against specific antigenic
variants of IBDV by incorporating the specific amino acid
changes in a mIBDV vaccine virus. Depending on the
composition, these mosaic IBD viruses (mIBDV) possess

25   different phenotypes and different antigenic properties.
A specific mutation in one of the viral proteins can have
a profound effect on IBDV viability. We found that this
is true in case of a single nucleotide substitution,
leading to a single amino acid mutation in VP4 (V582A).

30   No rIBDV could be rescued from cDNA when this particular
nucleotide substitution was present. Not only mutations
within the VP4 encoding region itself, but also mutations
or deletions in the region of the cleavage sites (pVP2-
VP4 and VP4-VP3) may have a negative effect on

35   replication of rIBDV. Mutations in the other viral
proteins, or even deletion of an entire viral protein
(i.e. VP5) influences the replication and or virulence as

1998). Apparently the VP5 protein, which is a non-structural protein, is also a non-essential protein. Yao et al. reported that inactivation of the ORF for VP5 (replacement of the startcodon by a stopcodon) yielded infectious rIBDV (rD78NS which grows to slightly lower titers (*in vitro*) than rD78, while Mundt et al. reported that inactivation of the ORF for VP5 (replacement of the startcodon by a arginine codon) yielded a rIBDV (IBDV/VP5-) which is able to grow to the same titers (*in vitro*) as the parental isolate. Furthermore Yao et al. reported that rD78NS has a decreased cytotoxic and apoptotic effects in cell culture (*in vitro*) and has a delay in replication compared to the parental isolate (*in vivo*), and failed to induce any pathological lesions or clinical signs of disease in infected chickens.

Mutations or deletions in the mIBDV cDNA yields a mIBDV with a desired phenotype, i.e. mIBDV which is based on a very virulent isolate but which has a reduced ability to replicate and hence an reduced pathogenicity. The introduction of cDNA sequences from a serotype II, cell culture adapted, IBDV isolate (TY89) into the mosaic virus gives us yet another opportunity to generate marker mIBDV vaccine which can be discriminated from wild-type serotype I IBDV, for example by using specific monoclonal antibodies. Such mIBDV can be used to induce an antibody spectrum, which differs from the spectrum induced by IBDV field isolates. This enables the development of a serologic test to determine whether IBDV antibodies are the result of live mIBDV vaccination or of infection with IBDV field isolates. For example, the mCEF94-s2VP3C virus is recognized by serotype II specific VP3 antibody (Mab T75) while it is also recognized by a serotype I specific VP2 antibody (Mab 1.4). This particular rIBDV is, on the

does not lead to major changes in replication ability in QM5 cells. When, on the other hand, the complete VP3 encoding region was exchanged we observed a severe reduction in replication ability of the resulting virus (mCEF94-s2VP3). On the other hand, mCEF94-s2VP3N was not reacting with Mab C3 (VP3, serotype I) while it is fully reacting with Mab B10 (VP3, serotype I) and only partially with Mab T75(VP3, serotype II). Replication of this mosaic IBDV on CEF cells is reduced compared to rCEF94. From the generated mIBDV, based on cDNA derived from serotype I (CEF94) and serotype II (TY89), it is clear that a serological marker based on VP3 has been identified. The replacement of the cDNA of (part of) VP3 of serotype I for the corresponding part of serotype II, leads to an unique combination of IBDV antigens within one mIBDV isolate. An mIBDV isolate based on this combination of antigens can be used as an IBDV-marker vaccine.

The introduction of the VP3 C-terminal part of TY89 (Serotype II) into the cDNA of D6948 yielded a mosaic IBDV (mD6948-s2VP3C1) which has a reduced virulence (no mortality, no body weight loss) compared to D6948 or rD6948 (Table 6). This mIBDV, or a comparable isolate which is more or less virulent, is also advantageously used as an IBDV marker vaccine to prevent infections with very virulent IBDV field isolates.

Furthermore, the invention provides using site-specific mutagenesis techniques to introduce any desired nucleotide mutation within the entire genome of mIBDV. Using this technique allows adapting mIBDV vaccines to future antigenic variations by including any mutation that has been found in antigenic variant IBDV field isolates. Furthermore, it is provided by the invention to exchange part of the genomic sequence of IBDV with the

IBDV or other Birna viruses. Also the use of cDNA of other Birna viruses (e.g. DXV, IPNV, OV or TV) leads to new IBDV vaccines. In this approach, one or more of the IBDV immunodominant or neutralizing epitopes are exchanged with the corresponding parts of the protein of another Birna virus.

Of course, the invention also provides a method for producing an rIBDV according to the invention, said vector comprising heterologous nucleic acid sequences derived from another virus, or (micro)organism, whereby r- or mIBDV serves as a vector. For example a method is provided to generate an infectious copy IBDV which expresses one or more antigens form other pathogens and which can be use to vaccinate against multiple diseases. Such an infectious copy IBDV for example comprises a heterologous cDNA encoding a heterologous protein obtained form a pathogen, for example poultry pathogens. Also a method is provided to generate a conditional lethal IBDV deletion mutant which can be used as self-restricted non-transmissible (carrier) vaccine. Such an IBDV deletion mutant is unable to express one of the IBDV proteins, and is phenotypically complemented by supplying the missing protein by other means.

The invention is further explained in the detailed description without limiting the invention thereto.

Material and Methods

5 Viruses and cells

The IBDV isolate CEF94 is a derivative of PV1 (Petek et al., 1973). After receiving the PV1 isolate in our laboratory in 1973, we have further adapted this isolated

10 by repeated passage (> 25 times) on either primary Chicken Embryo Fibroblast (CEF) cells or Bursa cells. The very virulent IBDV isolate D6948 was originally isolated in 1989 by the Poultry Health Service (Doorn, the Netherlands). It was purified by 5 passages in

15 embryonated eggs and one subsequent passage in SPF leghorn chickens. IBDV Serotype II isolate TY89 (McFerran et al., 1980) was maintained in our laboratory by a limited number of passages on CEF cells. Amplification of CEF-adapted isolates of IBDV (CEF94 and TY89) was

20 performed by growing freshly prepared chicken embryo fibroblast (CEF) cells in a tissue culture flask (75 cm2) until near confluency. This cell culture was infected with either CEF94 or TY89 (moi = 0.1) and incubated for 48 h at 37º C in a 5% CO_2 incubator. The supernatant of

25 this culture was centrifuged at 6000 g for 15 min. (pelleting of debris), transferred to clean tubes and subsequently centrifuged at 33.000 g for 3 h. The virus pellet was resuspended in PBS (1% of the initial culture volume). The very virulent IBDV isolate D6948 was

30 propagated in our laboratory in 21-days-old chickens by inoculation of 200 ELD50 (Egg Lethal Doses) per chicken, nasally and by eye-drop. The bursas of Fabricius were collected from the infected chickens three days post infection, and two volumes of tryptose phosphate buffer

35 was added. This mixture was homogenized in a Sorval Omnimixer (3 * 10 sec, maximum speed) and subsequently clarified by centrifugation (6000 g, 10 min). The

cells (Antin and Ordahl, 1991) were received from the laboratory of R. Duncan (Dalhouse University, Halifax,

5 Nova Scotia, Canada) and maintained by using QT35 medium

(Fort Dodge), in a CO_2 incubator (37° C).

Isolation of viral dsRNA

10 The genomic dsRNA was purified from the IBDV particles by digesting the viral proteins with Proteinase K (Amresco,

1.0 mg/ml) in the presence of 0.5 % SDS during 2 h at 50° C. The viral dsRNA was purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction

15 (two times) and precipitation with ethanol (2.5V) / NaCl (0.1V, 5M, pH4.8) or with 2 M lithiumchloride (Diaz-Ruiz and Kaper, 1978). The RNA was dissolved in DEPC treated

water (10 % of the initial volume) and stored at -20° C until further use. The integrity and purity of the viral

20 RNA was checked on an agarose gel.

Rapid Amplification of cDNA ends

The extreme 5'-termini of all genomic RNA strands (the
25 coding and non-coding strands of both the A- and B-segment) of isolate CEF94 were determined. We used 2 ug of genomic dsRNA and 10 pmol of strand- and segment specific primers in a total volume of 12 ul, for each

determination. After incubation at 95° C for five min. we
30 transferred this mixture onto ice and added 4 ul of Superscript II first strand syntheses buffer (Gibco/BRL), 2 ul of 100 mM DTT and 2 ul of dNTP's (10 mM each). This

mixture was subsequently incubated at 52° C for 2 min, after which 1 ul of reverse transcriptase (Superscript

35 II, Gibco/BRL) was added and incubation at 52° C was

neutralization, 2 ul of 6 M Acetic acid was added, and
cDNA was recovered by using a Qiaex DNA extraction kit
(Qiagen) and finally dissolved in 6 ul water. In the
anchor ligation reaction we used 2.5 ul of the cDNA
preparation, 4 pmol of the anchor, 5 ul T4 ligation
buffer and 0.5 ul T4 RNA ligase (New England Biolabs).
Incubation was performed at room temperature for 16 h and
the reaction was stored at - 20° C. To amplify the single
stranded cDNA which was ligated to the anchor, we used a
nested primer in combination with the anchor primer. The
PCR was performed by using the following conditions: 10
pmol of each specific primer, 10 pmol of the anchor
primer, 4.5 or 5.5 mM $MgCl_2$, 1* Taq buffer (Perkin
Elmer), 50 uM of each dNTP, 3 units of Taq polymerase
(Perkin/Elmer), and 4 ul of the RNA ligation mixture as
template, in a total volume of 50 ul. The amplification
was performed by 35 cycles through the temperature levels
of 92° C (45 sec), 57 or 65°C (45 sec), and 72°C (90
sec). The resulting PCR products were agarose gel
purified and digested with *EcoRI* and *XbaI* and ligated (T4
DNA Ligase, Pharmacia), in a pUC18 vector which had
previously been digested with the same restriction
enzymes. The resulting plasmids were amplified in *E. coli*
and nucleotide sequence analysis was performed by using
the M13F and M13R primers.

Generation of full length A- and B-segment single
stranded cDNA

To produce full length single stranded cDNA of both
the A- and B-segments of CEF94 and D6948, we used a
primer specific for the 3'-terminus of the coding strand
in the reverse transcription reaction for initiation of
the cDNA synthesis. As template we used 1 ug of genomic

and added 10 ul of RT-mix containing 2* Superscript II
first strand syntheses buffer (Gibco/BRL), 20 mM DTT, 2
5   mM of each dNTP and 100 units of Superscript II
(Gibco/BRL). In case of the negative control reaction the
addition of Superscript II enzyme was omitted. All tubes
were incubated at 50° C for 30 min, after which time 0.5
units of RNase H were added and incubation was continued
10   at 37° C for 15 min. Water (80 ul) was added to each
tube, and dsRNA and cDNA was purified by a
phenol/chloroform/isoamylalcohol (25:24:1) extraction and
precipitated by using a standard ethanol/NaAc
precipitation protocol. Obtained pellets were dissolved
15   in 20 ul of water and stored at -20° C.

Amplification of full length cDNA using a PCR based
protocol

20   The full length single stranded cDNA of both the A-
and B-segment were amplified by using PCR. The primers
which hybridize to the 3'-terminus of the non-coding
strand of the A-segment (T7AC0, Table 2) and B-segment
(T7BC1, Table 2) both have a non-hybridizing 5' extension
25   of 24 nt containing a T7 promoter sequence and an *Eco*RI
site. The primers that hybridize to the 5' terminus of
the coding strand of the A-segment (ANC0, Table 2) and B-
segment (BNC1, Table 2) match exactly. As template we
used 5 ul of the above mentioned RT reaction and the PCR
30   was performed in the presence of 1* Expand High Fidelity
buffer, 50 uM of each dNTP, 0.2 pmol of each primer, 1.5
units of Expand High Fidelity enzyme, and 2.0 mM MgCl2
(A-segment) or 4.0 mM MgCl2 (B-segment). Amplification
was performed by cycling 35 times between 94° C (15 sec),
35   58° C (15 sec) and 72° C (5 min) in case of A-segment

checked on a 1.0% agarose gel.

Cloning and analysis of the generated PCR fragments

The full length PCR fragments which were generated three
times independently from genomic dsRNA, were isolated
from the agarose gel by using a Qiaex gel purification
kit (Qiagen) and ligated in the pGEM-Teasy (Promega)
vector according to the suppliers instructions. The
ligated plasmids were used to transform *E. coli* DH5-alpha
cells which were subsequently grown under ampicillin
selection (100 ug/ml) and in the presence of IPTG (0.8 mg
per petri-dish)) and Bluo-gal (0.8 mg per petri-dish).
Plasmid DNA of white colonies was prepared and analyzed
by restriction enzyme digestion and agarose gel
separation. The nucleotide sequences of the cloned cDNA's
were determined by using a ABI310 automated sequencer and
A- and B-segment specific primers. The consensus
nucleotide sequences of both segments of CEF94 and of
both segments of D6948 were determined (Fig. 2) and the
corresponding amino acid sequence of the open reading
frames was deduced (Fig. 3). By using the cDNA of two
independent clones we restored one amino acid mutation
present in the A-segment clone (V542A), resulting in pHB-
36W, one amino acid mutation in the A-segment clone of
D6948 (P677L), resulting in pHB-60, and one amino acid
mutation in the B-segment of D6948 (Q291X), resulting in
pHB-55. No amino acid mutations were present in the B-
segment cDNA clone of CEF94 (pHB-34Z).

Introduction of a Hepatitis Delta Virus ribozym

The Hepatitis Delta Virus ribozym was first introduced
into the *E. coli* high copy number plasmid pUC-18 by

was ligated in the pUC18 vector which previously was digested with *Xba*I and *Sma*I, yielding pUC-Ribo. Plasmids

5   containing the A- and B-segment of CEF94 and D6948 were used as template in a full length PCR using the above described conditions, and primers specific for either the A-segment (T7AC0 and ANC0) and B-segment (T7BC1 and BNC1). The PCR fragments were agarose gel purified

10 (Qiaex), blunt-ended by using T4 DNA polymerase, and subsequently digested with *Eco*RI. The resulting DNA fragments were directionally cloned into the pUC-Ribo vector which previously had been digested with *Sma*I and *Eco*RI. The resulting plasmids were used as template in an

15 *in vitro* transcription-translation reaction (TnT-T7Quick, Promega). The autoradiogram of SDS-PAGE analyses of the translation products revealed three dominant bands pVP2 (48-49 kDa), VP3 (32-33 kDa), and VP4 (28-29 kDa)) when pHB-36A (A-segment of CEF94) or pHB-60 (A-segment of

20 D6948) was used as template. One dominant band (VP1 (91 kDa)) was found when we used plasmid pHB-34Z (B-segment of CEF94) or pHB-55 (B-segment of D6948) as template (data not shown).

25 Introduction of a genetic tag

To distinguish infectious virus generated from cloned cDNA from wild-type virus we introduced a genetic tag in the 3'-UTR of the A-segment of IBDV-A isolate. Two

30 nucleotides of pHB-36A were mutated (C3172T and 3T173A) thereby introducing a unique *Kpn*I restriction site (GGTAAC). These mutations were introduced by the method described by Higuchi (1990). A 383 bp fragment of the resulting PCR fragment was ligated (Rapid ligation kit,

35 Boehringher Mannheim) into the full length A-segment clone (pHB-36A) by using two unique restriction sites

from sequence analysis and digestion with restriction enzyme *KpnI* (data not shown). No difference was observed

5   in the resulting protein pattern when either pHB-36A or pHB-36W was used as template in an *in vitro* transcription/translation reaction (data not shown).

Construction of mosaic A-segment cDNA

10

We constructed plasmids containing mosaic IBDV A-segments which partly consisted of cDNA of one isolate (CEF94) and partly of cDNA of another isolate (D6948). To construct these plasmids we have amplified specific parts of cDNA

15   using appropriate IBDV specific or selective primers. The amplified PCR fragment of cDNA of D6948 was subsequently used to replace the corresponding part in plasmids pHB-36W, using restriction endonucleases and T4 DNA ligase (Rapid DNA Ligation, Boehringher Mannheim).

20   For the construction of pHB36-vvVP2 (exchange of pVP2 encoding part, Table 4) we have used IBDV specific to generate the mosaic PCR-VP2D fragment (2256 bp, see Fig. 5a). The internal part of this PCR fragment was used to exchange the corresponding part of pHB-36W, using

25   unique sites for restriction enzymes *EcoRI* and *SacII*.

For the construction of plasmid pHB36-vvVP3 (Table 4) we used IBDV specific primers to generate a mosaic PCR-VP3c fragment (2154 bp, see Fig. 5b). The internal part of this PCR fragment was used to exchange the corresponding

30   part of pHB-36W, using unique sites for the *EagI* and *KpnI* (genetic tag site) restriction enzymes.

For the construction of plasmid pHB36-vvVP4 (Table 4) we used IBDV specific primers to generate a mosaic PCR-VP4d fragment. (2154 bp, see Fig. 5c). The internal part of

35   this PCR fragment was used to exchange the corresponding part of pHB-36W, using the unique site for restriction

that no unintended mutations were introduced during the
described manipulations.

Introduction of a serological marker

To obtain the cDNA of the A-segment of a serotype II
IBDV isolate we generated single stranded cDNA of TY89 as
described above, by using the ANC1 primer. The coding
region of the VP3 protein was subsequently three times
independently amplified in a PCR by using 2 ml of RT-
material, 1* Taq buffer, 50 uM of each dNTP, two IBDV
serotype II specific primers (0.2 pMol each), 1.5 units
of enzyme, and 3.0 mM $MgCl_2$ in a 0.1 ml reaction volume.
Amplification was performed by cycling 35 times between
94° C (15 sec), 52° C (15 sec) and 72° C (1 min). The
resulting 956 bp fragment was cloned in the pGEM-TEasy
vector and the consensus nucleotide sequence was
determined (Fig. 2a). One of the isolated plasmids
contained the TY89 VP3 consensus sequence (pSV-VP3-TY89,
Fig. 4) and was used as template to generate a 893 bp PCR
fragment (see Fig. 5d). This PCR fragment was
subsequently used to replace the corresponding part of
plasmid pHB36W-vvVP3, by using the artificially
introduced KpnI (nt 3175) and SacII (nt 1760) restriction
sites in both plasmid pSV-VP3-TY89 and pHB36W-vvVP3. The
resulting plasmid (pHB36-vvVP3, see Fig. 5d) encodes the
N-terminal 722 amino acids of the CEF94 polyprotein and
the 290 C-terminal amino acids of the TY89 polyprotein.
The intended exchange was confirmed by nucleotide
sequence analysis.

The same approach was used to exchange the C-terminal
half of the coding sequence of the VP3 protein. In stead
of the artificially introduced SacII site, we made use of

encodes a polyprotein consisting of the N-terminal 890
amino acid of the CEF94 polyprotein, in combination with
the C-terminal 122 amino acids of the TY89 polyprotein.

For the construction of plasmid pHB36-s2VP3N (see Table
4) we have replaced the ScaI (nt 2799) – KpnI (nt 3172)
part of plasmid pHB36-s2VP3 with the corresponding part
of plasmid pHB-36W. Using the specific restriction
endonucleases ScaI and KpnI, and T4 ligase. The
nucleotide sequence of plasmid pHB36-s2VP3N was conformed
by sequence analysis.

For the introduction of the C-terminal encoding part of
the VP3 protein of IBDV isolate TY89 into the cDNA of
isolate D6948 we have exchanged part of plasmid pHB-60
(nt 1760 -> nt 3260) with the corresponding part of
plasmid pHB36-s2VP3C. Plasmid pHB36-s2VP3C was digested
with restriction enzymes SacII and XbaI and a 1735 bp
fragment was recovered from an agarose gel by Qiaex gel
extraction kit (Qiaex). This DNA fragment was ligated in
the 4440 bp vector fragment of pHB-60 which had
previously been digested with the same restriction
enzymes. The resulting plasmid (pHB60-s2VP3C1, Table 4)
contains cDNA derived from IBDV isolate D6948 (nt 1 to
1760), CEF94 (nt 1760 to 2799 and nt 3175 to 3260), and
TY89 (nt 2799 to 3175).

Transfection of QM5 cells

QM5 cells, grown to 80% confluency in 60 mm dishes,
were infected with Fowl Pox T7 (FPT7) (Britton et al.,
1996) one hour prior to transfection. FPT7 infected QM5

supplemented with 25 ul LipofectAMINE (Gibco/BRL) and kept at room temperature for at least 30 min. The washed

5   QM5 cells were covered with 4 ml of Optimem 1, the DNA/LipofectAMINE transfection mixture was added and the cells were stored for 18h in a 5.0% CO_2 incubator at 37°C.

10 Detection of recombinant IBDV after transfection of QM5 cells

Transfected QM5 cells were washed once with PBS after the transfection. Infectious recombinant IBDV (rIBDV) was

15 recovered from transfected QM5 cells by covering them with 4 ml of QT-35 medium supplemented with 5% fetal calf serum and 2% of an antibiotic mix (1000 U/ml Penicillin, 1000 ug/ml Streptomycin, 20 ug/ml Fungizone, 500 ug/ml; Polymixin B, and 10 mg/ml Kanamycin) and incubation for

20 24 h at 37° C (5.0% CO_2). The supernatant was filtered through a 200 mM filter (Acrodisc) to remove FPT7 virus and was subsequently stored at -70° C or used directly for quantitation of rIBDV. Recombinant mosaic IBDV (mIBDV) which contains at least the pVP2 from vvIBDV

25 isolate D6948 is unable to re-infect QM5 cells (see Table 5). Therefore, supernatant of transfection experiments which contained D6948 pVP2 encoding cDNA were used to infect 11-days-old, embryonated eggs via the chorioallantoic membrane (CAM) route. To determine the

30 presence of infectious IBDV, the embryo's were collected five days post-infection, homogenized by using a Sorval Omnimixer (3 * 10 sec, max. speed) and assayed for the presence of IBDV proteins in a IBDV protein specific Elisa.

35

Different monoclonal antibodies were used to detect
recombinant mosaic IBDV (mIBDV) that contained part of
the TY89 VP3 or the complete TY89 VP3. The mIBDV's were
used to infect QM5 or primary bursa cells and incubated
for 24h (QM5 cells) or 48h (primary bursa cells) in a 5%
CO_2 incubator at 37° C or 39° C, respectively. The
infected cells were subsequently fixed and an
immunoperoxidase monolayer assay (IPMA) was performed by
using monoclonal antibodies which are either specific for
VP2 of serotype I IBDV (Mab 1.4), or specific for VP3 of
serotype II (Mab T75), or specific for VP3 of serotype I
(Mab B-10 or C-3).

Virulence of rIBDV in young SPF chickens

To evaluate the degree of virulence of the generated
rIBDV, srIBDV, and mIBDV isolates we have inoculated 12
groups (10 21-days old SPF chickens) with these viruses.
Each chicken received nasally and by eye-drop 1000 ELD50
IBDV, with exception with the negative control group,
which received only PBS. The animals were monitored for
clinical signs and dead chicks were removed each day. At
9 days post infection, all the chicks from the negative
control groups and all the surviving chicks from groups
in which mortality had occurred, were bled (5 ml) and
euthanized for necropsy. From the other groups, 6 chicks
were bled (5 ml) and taken for necropsy at day 9 post
infection, where as the remaining 4 were bled (5 ml) and
taken for necropsy at day 15 post infection. Bursa and
body weight was determined of all chicks which had been
euthanized at day 9 post infection

Nucleotide sequence determination of the 5'-termini.

5   One group has reported the 5'- and 3'-terminal sequences
     of the segmented dsRNA genome of IBDV (Mundt and Muller,
     1995). To verify the terminal sequence of the genome of
     IBDV and to be able to produce the exact cDNA sequence of
     a single IBDV isolate we have determined the 5' terminal
10   sequences of both the coding and non-coding strands of
     the two genomic segments of CEF94, a Chicken Embryo
     Fibroblast (CEF) adapted, classical isolate of IBDV, by
     using the RACE (Rapid Amplification of cDNA Ends)
     technique (Frohman et al., 1988). The RACE analysis was
15   performed in duplicate on each of the four 5'-termini of
     the CEF94 genome. The resulting sequence data (Table 3)
     show that the length of the 5'-termini of the coding
     strands was the same in all cases. Furthermore we found
     that the nucleotide sequence was identical, except for
20   the last nucleotide which varied in a few clones. This is
     in contrast to the sequence data of the 5'-termini of the
     non-coding strands, which varied in length considerably.
     We also found that the last nucleotide, although
     preferably a cytosine, varied in some clones similarly to
25   what we found for the 5'-termini of the coding strands.
     The consensus sequence for the 3'-terminal nucleotide of
     the A-segment coding strand of CEF94 differs from the
     nucleotide sequence reported by Mundt and Muller (Mundt
     and Muller, 1995), i.e. being a cytosine in stead of a
30   thymine.

Generation of plasmids containing full length IBDV cDNA

         Using the sequence data of the 5'-termini we cloned
35   the entire coding and non-coding cDNA sequences of the A-
     segment and B-segment of classical isolate CEF94 by means
     of RT-PCR. Using the same procedure and using the same

three times independently. This sequence information
enabled us to generate a consensus nucleotide sequence of
5   both the A- and B-segments of IBDV isolates CEF94 and
D6948 (Fig. 2A).

Fowlpox T7 polymerase expression system

10   One system for generating infectious IBDV virus
using *in vitro* synthesized mRNA derived from cDNA of a
CEF-adapted IBDV isolate has previously been described
(Mundt and Vakharia, 1996). This system is based upon *in
vitro* run-off transcription from the T7 promoter which
15   was artificially introduced in front of the cDNA
sequences of the A- and B-segments. This RNA is
subsequently transfected into VERO cells, after which
infectious IBDV virus could be harvested from these
cells. One of the drawbacks of this system is that the *in
20   vitro* generated RNA has to contain a 3'-G-ppp5'- (cap
structure) on it's 5'-end in order to get translation of
the introduced RNA into the viral proteins, and hence
replication of viral RNA. The *in vitro* production of high
quality mRNA is both inefficient and expensive as a cap
25   structure has to be present at the 5'-end. Furthermore,
expression levels from transfected RNA are generally low
due to the short half-life of RNA. To circumvent the
drawbacks of generating *in vitro* capped RNA and low
expression levels, we have explored the possibility of
30   using an *in vivo* based T7-expression system (Fowlpox T7
polymerase expression system, (Britton et al., 1996) for
generation of viral RNA from plasmids containing full
length IBDV cDNA.

To be able to generate IBDV from cloned cDNA which
5   has the authentic terminal sequences, we introduced the
cis-acting Hepatitis Delta Virus (HDV) ribozym (Chowrira
et al., 1994) downstream of the cDNA sequence of the A-
and B-segments (Fig. 4). Furthermore we introduced an
additional modification in 3' untranslated region of the
10   CEF94 A-segment. By exchanging 2 nucleotides we
introduced a unique KpnI endonuclease restriction site in
this cDNA. The introduction of this unique restriction
site enables us to distinguish between wild-type IBDV and
infectious IBDV virus generated from cloned cDNA
15   (genetically tagged rIBDV). As expected, this plasmid
yields the same viral proteins in an in vitro
transcription-translation reaction as the A-segment clone
without the genetic tag (data not shown).
Plasmid pHB-36W (A-segment CEF94), pHB-60 (A-segment
20   D6948), pHB-34Z (B-segment CEF94), and pHB-55 (B-segment
D6948) were used individually to transfect FPT7 infected
QM5 cells as described in the Materials and Method
section. To analyze whether the transfected QM5 cells
expressed IBDV proteins, we performed an IPMA, 24 h after
25   transfection. We used polyclonal antiserum directed
either against VP3 (pHB-36W and pHB-60 transfections) or
VP1 (pHB-34Z and pHB-55 transfections) in this analysis.
About 10 to 50% of the QM5 cell expressed VP3 after
transfection with pHB-36W or pHB-60 (data not shown).
30   When B-segment encoding plasmids were used (pHB-34Z or
pHB-55) we found that the same percentage of cells (about
10 to 50%) were expressing VP1 (data not shown).
Subsequently, we co-transfected combinations of plasmids
containing the A- and B-segment cDNA's into FPT7 infected
35   QM5 cells. To screen for infectious recombinant IBDV
(rIBDV) in the supernatant of the transfected QM5 cells,

used to transfect the QM5 cells. rIBDV could not be
detected when supernatant of the cells transfected with
A-segment (pHB-60) and B-segment (pHB-55) of D6948
(rD6948) was transferred onto QM5 cells. However, when
the co-transfection supernatant of pHB-60 and pHB-55 was
transferred onto primary bursa cells or embryonated eggs
we were able to show the presence of infectious IBDV
(rD6948) in primary bursa cells (after 48h) and in
embryonated eggs (after five days). The presence of rIBDV
in the first passage was established by using either an
IPMA (QM5 cells or primary bursa cells) or an IBDV
specific Elisa (embryonated eggs). The generated rCEF94
and rD6948 isolates were amplified in 10-days old
embryonated SPF eggs and subsequently used to infect 21-
days old SPF chickens (10 chickens per IBDV isolate). The
resulting data of the animal experiment (Table 6) shows
that the mortality, body weight, bursa weight, and bursa-
body weight ratio, caused by rD6948 are the same as the
parent very virulent D6948 isolate. Also at necropsy,
gross lesions of bursa were as severe for rD6948 as for
the parental D6948 isolate (data not shown). From this
chicken experiment it is concluded that rD6948 has
retained the properties of a very virulent IBDV isolate,
and is truly a very virulent rIBDV.

Detection of the genetic tag

Supernatant of rCEF94 infected QM5 cells was harvested
and IBDV was isolated by centrifugation as described in
the material section. The dsRNA genome was extracted and
an A-segment specific primer was used to generate single
stranded cDNA, by using reverse transcriptase. The cDNA
was subsequently amplified by PCR. The generated PCR
fragment was cloned into a high copy number *E. coli*
plasmid (pGEM-Teasy, Promega) and was either digested

Identification of a lethal amino acid mutation in VP4

Plasmid pHB-36 (A-segment CEF94, Table 4) contained a single nucleotide substitution at position 1875 (thymine in stead of a cytosine) compared to the consensus CEF94 A-segment sequence (Fig. 2A). This nucleotide substitution leads to a valine at position 582 of the polyprotein in stead of an alanine, which is encoded by the consensus sequence (V582A, Fig. 3A). As this amino acid mutation is present in the viral protease (VP4), we subsequently checked whether this protease was still able to autocatalytically liberate the viral proteins (pVP2, VP3 en VP4) from the polyprotein. When plasmid pHB-36 was used as template in a coupled in vitro

transcription/translation reaction in the presence of ^{35}S labeled methionine we found a delayed splicing of the polyprotein (data not shown). Apart form the viral proteins which are found in case of normally spliced polyprotein (pVP2, VP3 and VP4), we found intermediate spliced products (60 kDa: VP4+VP3), and non-spliced polyprotein (data not shown). Although the viral protease (VP4) of clone pHB-36 is able to liberate the structural viral proteins (pVP2 and VP3) from the polyprotein, this clone did not yield rIBDV when using the FPT7 based transfection protocol as described above. Rapid autocatalytic cleavage of the polyprotein is apparently necessary for the generation of infectious rIBDV. We expect that other mutations within VP4 which alter the rate or specificity of the autocatalytic cleavage of the polyprotein will also have a negative effect on viability of the generated rIBDV. Furthermore mutations in the region of the cleavage sites (pVP2-VP4 and VP4-VP3) may also have a negative effect on replication of rIBDV. Any

Generation of segment reassortant IBDV

Transfection of CEF94 A-segment cDNA (pHB-36W) in combination with D6948 B-segment cDNA (pHB-55) yielded segment reassorted IBDV (srIBDV-CADB) when supernatant of QM5 transfected cells was transferred onto fresh QM5 cells (Table 5). When D6948 A-segment cDNA (pHB-60) was used in combination with CEF94 B-segment cDNA (pHB-34Z) no infectious srIBDV (srIBDV-DACB) could be detected on QM5 cells (Table 5). However, when primary bursa cells were used to assay for the presence of infectious IBDV we found in both cases (srIBDV-CADB and srIBDV-DACB) infected cells after 24h of incubation. Out of the population of primary bursa cells, only lymphoid cells were infected with srIBDV-DACB, while both lymphoid and fibroblast cells were infected in the case of srIBDV-CADB. The srIBDV-DACB isolate induces the same clinical signs as D6948, while the srIBDV-CADB isolate has a comparable virulence as CEF94 (Table 6).

Construction of mosaic IBDV

By using modern molecular biological techniques such as those described above, we have created mosaic recombinant IBDV (mIBDV) which exists partly of cDNA derived from CEF94, and partly from D6948 (vvIBDV) or TY89 (a serotype II IBDV isolate). Replacement of the pVP2 protein encoding sequence of CEF94 by the corresponding part of the D6948 yielded only virus (mCEF94-vvVP2) when the supernatant of transfected cells was transferred to cells which are normally susceptible for non CEF-adapted vvIBDV, i.e. primary bursa cells or embryonated eggs. (Table 5). Replacement of the VP3 or VP4 protein encoding sequence of CEF94 with the corresponding part of D6948

cDNA yielded a plasmid which encoded a polyprotein consisting of pVP2 and VP4 derived from CEF94 and of VP3 derived from TY89. When this plasmid (pHB36-s2VP3) was used in an in vitro transcription-translation reaction, all the expected proteins, pVP2, VP4 and VP3 were present (data not shown). Transfection of this plasmid in combination with a plasmid (pHB-34Z) containing the B-segment cDNA of CEF94 yielded a mosaic IBDV (mCEF94-s2VP3). Two monoclonal antibodies which are specific for serotype I VP3 (Mab B10 and C3) were unable to recognize this mCEF94-s2VP3, while an antibody which is specific for the serotype II VP3 (Mab T75) did recognize this mosaic virus (Table 5). As expected the mCEF94-s2VP3 was also recognized by a serotype I specific, neutralizing monoclonal antibody directed against VP2 of the CEF94 isolate (Mab 1.4). The TCID50 on QM5 cells, which was determined 18 hours after transfection, was considerably lower (3 logs) in the case of mCEF94-s2VP3 compared to rCEF94. Furthermore we found that only single QM5 cells were infected by mCEF94-s2VP3 after 24 h. This is in contrast to the plaque forming phenotype of CEF94 and rCEF94 on QM5 cells after 24 h of infection. To generate mIBDV which has the same replication and plaque forming characteristics as rCEF94, but which is still antigenetically different from rCEF94 we subsequently exchanged only the N-terminal part (168 amino acids) or C-terminal part (122 amino acids) of the VP3 of CEF94 by the corresponding sequence of TY89. When these mosaic A-segment plasmid (pHB36-s2VP3N or pHB36-s2VP3C) were transfected in combination with pHB-34Z (CEF94 B-segment) we obtained mosaic IBDVÆs (mCEF94-s2VP3N or mCEF94-s2VP3C) with replication capabilities in QM5 cells that

infected cells (Table 5). Mab T75 which is specific for
VP3 of serotype II also recognized mCEF94-s2VP3C, while
5   the recognition of mCEF94-s2VP3N was slightly reduced.
Mab B10, which is specific for VP3 of serotype I did not
recognize rCEF94-s2VP3C, while it still recognized
mCEF94-s2VP3N. Another Mab (C3) which did not react with
mCEF94-s2VP3 infected cells did react with mCEF94-s2VP3C
10 infected cells, although the reaction was reduced
compared to QM5 cells infected with rCEF94 (Table 5)
mCEF94-s2VP3N was not recognized by Mab C3. The serotype
I specific, neutralizing antibody Mab 1.4 which
recognizes VP2 recognized, as expected, both mCEF94-
15 s2VP3N and mCEF94-s2VP3C.

The coding sequence of the C-terminal part of
serotype II VP3 (122 amino acids) was also used to
replace the corresponding part of the cDNA of D6948.
During the exchange we have replaced some D6948 cDNA
20 sequence (encoding for C-terminal part of VP4 and the N-
terminal part of VP3, and the 3'-UTR) with the
corresponding sequence of CEF-94 (see Fig. 5g). The
resulting plasmid (pHB60-s2VP3C1) was, together with pHB-
55 (B-segment D6948), transfected into FPT7 infected QM5
25 cells. Supernatant of these transfected QM5 cells was
collected after 24 h and was transferred to embryonated
eggs and primary bursa cells. By using monoclonal
antibodies we were able to detect infected cells in the
monolayer of primary bursa derived cells (see Table 5).
30 mD6948-s2VP3C1 gave the same reaction pattern with the
monoclonal antibodies as mCEF-s2VP3C did. Isolate mD6948-
s2VP3C1 (1000 ELD50/chicken) was also used to infect 10
SPF chickens (21-days old) to evaluate its virulence.
This mIBDV isolate did not cause any mortality in a 9-
35 days course, opposite to the D6948, rD6948 and srIBDV-
DACB isolates (Table 6). However, the bursa is severely

of Fabricius.

Fig. 1:   Antibody titers in broilers having high levels of maternal antibody at day 0.

Table 1: Classification of live IBDV vaccines used to induce active protection in young chickens which are passively protected by maternal IBDV antibodies.

Type of vaccine (live IBD virus)	Ability to induced an immune response when IBDV antibody titers are equal or below	Immunosuppressive
Mild	50-100	No
Intermediate	100-200 *	No
Strong	500 *	Yes

* The Animal Health Service (Deventer, The Netherlands) uses an Idexx Elisa value of 128 (2log7) as the maximum titer for the use of live intermediate vaccins and a value of 512 (2log9) for strong vaccines.

which are unable to hybridize with wild-type IBDV genomes
are given in small face. Primers which are specific

5   either for the serotype II (s2) or very virulent (vv)
genome are indicated.

Name	Sequence	Position
Anchor	cacgaattcactatcgattctggatccttc	-
Anchor Primer	gaaggatccagaatcgatag	-
ANC0	GGGGACCCGCGAACGGATC	A: -1/-18
ANC1	GGGGACCCGCGAACGG	A: -1/-16
T7AC0	ggaattctaatacgactcactataGGATACGATCGGTCTGACCCCGG	A: 1/23
BNC1	GGGGGCCCCCGCAGG	B: -1/-15
T7BC1	ggaattctaatacgactcactataGGATACGATGGGTCTGACCCT	B: 1/21

10

3   Nucleotide sequence corresponding to the 5'- and 3'-termini of the coding strands (
the two genomic segments of IBDV (CEF94). Numbers behind specific sequences indica
the number of times each sequence was obtained.

5'-terminus of the A-segment coding strand	Complementory sequence of the 5'-terminus of the A-segment non-coding strand	5'-terminus of the B-segment coding strand	Complementory sequenc( ) of the 5'-terminus of B-segment non-coding
5'UGAUACGAUC>>>	>>>CGGG3'	5'UGAUACGAUG>>> (2x)	>>>GGGGGCCA3'
5'AGAUACGAUC>>>	>>>CGGGUCCC3'	5'GGAUACGAUG>>> (5x)	>>>GGGGGCCU3'
5'GGAUACGAUC>>> (7x)	>>>CGGGUCCCU3'		>>>GGGGGCCC3' (2:
	>>>CGGGUCCCC3' (6x)		>>>GGGGGCCCC3' (2:
	>>>CGGGUCCCCC3'		>>>GGUGGCCCCC3'
	>>>CGGGUCCCCCU3'		>>>GGGGGCCCCC3'
	>>>CGGGUCCCCCC3'		>>>GGGGGCCCCG3'

nsus 5'GGAUACGAUC>>>   >>>CGGGUCCCC3' (nt 3260) 5'GGAUACGAUG>>>   >>>GGGGGCCCCC3' (nt

4   Description of the used plasmids

	Based on plasmid	Description
ibo	pUC18	Contains the *SmaI-XbaI* fragment of pTV-2A
	pUC18-Ribo	Contains the consensus cDNA sequence of the A-segment of CEF94 (see Fig. 2a)
	pHB-36A	An artificially introduced *KpnI*-site (genetic tag) in the 3'-UTR of the CEF94 A-segment encoding cDNA (Fig.
	pHB-36A	Contains a lethal amino acid substitution in the VP4 part of the polyprotein (V582A)
	pUC18-Ribo	Contains the consensus cDNA sequence of the D6948 A-segment (see Fig. 2a)
	pUC18-Ribo	Contains the consensus cDNA sequence of the CEF94 B-segment (see Fig. 2b)
	pUC18-Ribo	Contains the consensus cDNA sequence of the D6948 B-segment (see Fig. 2b)
9-VP3	pGEM-Teasy	Contains the consensus cDNA of TY89 encoding the entire VP3 (A-segment, see Fig. 2)
vVP2	pHB-36W	Contains D6948 A-segment cDNA which encodes the entire VP2 (453 amino acids)
vVP3	pHB-36W	Contains D6948 A-segment cDNA which encodes the entire VP3 (289 amino acids)
vVP4	pHB-36W	Contains D6948 A-segment cDNA which encodes the entire VP4 (270 amino acids)
2VP3	pHB-36W	Contains TY89 A-segment cDNA which encodes the entire VP3 (289 amino acids)
2VP3C	pHB-36W	Contains TY89 A-segment cDNA which encodes the C-terminal part (122 amino acids) of VP3
2VP3N	pHB-36W	Contains TY89 A-segment cDNA which encodes the N-terminal part (168 amino acids) of VP3
2VP3C1	pHB-60	Contains cDNA encoding a mosaic polyprotein (D6948 (1-543 AA), CEF94 (544-889 AA), and TY89 (890-10 AA). The 5'-UTR is derived from D6948, while the 3'-UTR is derived from CEF94. An unique *KpnI*-site (gene is furthermore present in the 3'-UTR

5   Description of the generated rIBDV, srIBDV, and mIBDV. The ability of these viruses
: QM5 or primary bursa cells was examined in an immuno peroxidase monolayer assay (IPMA)
either polyclonal serum directed against VP3 or monoclonal antibodies directed against
: IBDV serotype I (1.4), VP3 of serotype II (T-75), or VP3 of serotype I
and C3); nd means not determined.

Virus	Derived from plasmids		Replication on		Mab's			
	A-segment	B-segment	QM5 cells	Bursa	1.4	T75	B10	C3
1	pHB-36W	pHB-34Z	+	+	+	-	+	+
3	pHB-60	pHB-55	-	+	+	-	+	+
-DACB	pHB-60	pHB-34Z	+	+	nd	nd	nd	nd
-CADB	pHB-36W	pHB-55	-	nd	nd	nd	nd	nd
1-vvVP2	pHB36-vvVP2	pHB-34Z	+	+	nd	nd	nd	nd
1-vvVP3	pHB36-vvVP3	pHB-34Z	+	nd	nd	nd	nd	nd
1-vvVP4	pHB36-vvVP4	pHB-34Z	+	nd	nd	nd	nd	nd
1-s2VP3	pHB36-s2VP3	pHB-34Z	+	nd	+	+	-	+/-
1-s2VP3C	pHB36-s2VP3C	pHB-34Z	+	nd	+	+	-	+/-
1-s2VP3N	pHB36-s2VP3N	pHB-34Z	+	nd	+	+/-	+	-
3-s2VP3C1	pHB60-s2VP3C1	pHB-55	-	+	+	+	-	+/-

Clinical data of 21-day old chickens infected with wild-type, rIBDV, srIBDV or mIBDV isolates (12 groups of 10 chickens). Each chicken was inoculated with 1000 ELD_{50} IBDV, expect for the negative control group (PBS), and each group was kept in a separate isolator. The bursa and body weight of euthanized chickens was determined at nine days post infection. Standard deviation is given between brackets, together with the number of animals (n) used for determination of the given numbers. The bursa/body weight ratio for each animal was calculated and mean values (standard deviation) per group are given.

us	Number of deads (after 9 days)	Body weight (grams)	Bursa weight (grams)	Bursa/Body weight (*1000)
	0	305 (29, n=10)	1.9 (0.4, n=10)	6.1 (1.2)
	0	341 (16, n=6)	2.0 (0.6, n=6)	6.0 (1.8)
	3	245 (56, n=7)	0.4 (0.1, n=7)	1.7 (0.6)
	0	317 (15, n=6)	1.3 (0.5, n=6)	4.2 (1.3)
	5	261 (24, n=5)	0.4 (0.1, n=5)	1.7 (0.2)
DACB	2	263 (35, n=8)	0.4 (0.1, n=8)	1.5 (0.3)
CADB	0	314 (13, n=6)	1.8 (0.8, n=6)	5.7 (2.7)
-vvVP2	0	309 (27, n=6)	0.6 (0.2, n=6)	1.9 (0.4)
-vvVP3	0	325 (33, n=6)	2.0 (0.3, n=6)	6.2 (0.7)
-vvVP4	0	330 (23, n=6)	1.5 (0.5, n=6)	4.4 (1.3)
-s2VP3C	0	320 (11, n=6)	1.3 (0.4, n=6)	4.1 (1.3)
-s2VP3C1	0	315 (26, n=6)	0.6 (0.2, n=6)	1.9 (0.6)

origin and phenotype of the IBDV isolates

Reference	Virulence
Boot et al., unpublished	Very virulent
Boot et al., unpublished	Very virulent
Brown and Skinner, 1996	Very virulent
Ter Huurne et al., unpublished	Very virulent
Ter Huurne et al., unpublished	Avirulent
Ter Huurne et al., unpublished	Avirulent
Ter Huurne et al., unpublished	Avirulent
Ter Huurne et al., unpublished	Very virulent
Ter Huurne et al., unpublished	Avirulent
Ter Huurne et al., unpublished	Very virulent
Ter Huurne et al., unpublished	Very virulent
Ter Huurne et al., unpublished	Very virulent
Ter Huurne et al., unpublished	Very virulent
Ter Huurne et al., unpublished	Very virulent
Yamaguchi et al., 1996	Very virulent
Yamaguchi et al., 1996	Avirulent
Yamaguchi et al., 1996	Very virulent
Yamaguchi et al., 1996	Avirulent
Lim et al., 1999	Very virulent

Lim et al., 1999   Not determined

Amino acid sequence of the hypervariable region of VP2 of different IBDV isolates. The sequence of the hydrophilic regio (underlined) and the hydrophobic region of very virulent isolate D6948 (amino acid 214 to 328) is used as parental isolate 1 alignment of the other sequences. Identical amino acid are represented by a dash.

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214 ADDYQFSSQYQAGGVTITLFSANIDAITSLSIGGELVFQTSVQGLILGATIYLIGFDGTAVITRAVAADNGLTAGTDNLMPFNIVIPTSEITQPITSIKLEIVTSKSGGQAGDQMSWS 328
    -------------------------------------------------------------------------------------------------------------------
    -------------------------------------------------------------------------------------------------------------------
    -------P------------------------V----------T-----N----T------L---N-------------------------------------------------
    -------L------------------N-A-N---------T----T-----T-I-----N-------------------------------------------------------
    -------P------------------V-------------Y----T----L----N--------------------------------------------------------
    -----------------------------------------------N-----------------------------------------------------------------
    -------------------------------------------------------------------------T-----V--------------------------------
    ------------------------------------------------------------------------F---------------------------------------
    --------------------------------------T----------N---T-----------------------------------------------------------
    --------------------------H----D----------T-----N---T-----------------------------------------------------------
    ----------------------------------------------------N---T-----------------------------------------------------
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Antin, P.B. and Ordahl, C.P. (1991) Isolation and characterization of an avian myogenic cell line. Dev Biol 143(1), 111-21.

Berg, T.P.v.d., Gonze, M. and Meulemans, G. (1991) Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. Avian Pathology 20(1), 133-143.

Bernard, J. (1980) Drosophila X virus RNA polymerase: tentative model for in vitro replication of the double-stranded virion RNA. J. Virol. 33, 717-723.

Britton, P., Green, P., Kottier, S., Mawditt, K.L., Penzes, Z., Cavanagh, D. and Skinner, M.A. (1996) Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. J Gen Virol 77(Pt 5), 963-7.

Brown, M.D. and Skinner, M.A. (1996) Coding sequences of both genome segments of a European 'very virulent' infectious bursal disease virus. Virus Res. 40, 1-15.

Bruenn, J.A. (1991) Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. Nucleic Acids Res 19(2), 217-26.

Chettle, N.J., Stuart, J.C. and Wyeth, P.J. (1989) Outbreak of virulent infectious bursal disease in East Anglia. Veterinary Record 125(10), 271-272.

Chowrira, B.M., Pavco, P.A. and McSwiggen, J.A. (1994) In vitro and in vivo comparison of hammerhead, hairpin, and hepatitis delta virus self-processing ribozyme cassettes. J Biol Chem 269(41), 25856-64.

Cosgrove, A.S. (1962) An apperently new disease of chickens: avian nephrosis. Avian Diseases 6, 385-389.

De Wit, J.J. and Van Loon, A.A.W.M. (1998) Gumboro-vaccinatie. Tijdschrift voor Diergeneeskunde 123, 7-10.

Diaz-Ruiz, J.R. and Kaper, J.M. (1978) Isolation of viral

characterization of five animal viruses with bisegmented
double-stranded RNA genomes. J. Virol. 32, 593-605.

5   Ducatelle, R.V.A., Uyttebroek, E., De Ruyne, L., Mast,
J., Goddeeris, B., Desmidt, M. and Deherdt, P. (1995)
Infectious Bursal Disease in Europe, Consequences of
changing epidemiological conditions. Summit on Infectious
Bursal Disease, 10-14.

10 Eterradossi, N., Rivallan, G., Toquin, D. and Guittet, M.
(1997) Limited antigenic variation among recent
infectious bursal disease virus isolates from France.
Arch Virol 142(10), 2079-87.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Rapid
15 production of full-length cDNAs from rare transcripts:
amplification using a single gene-specific
oligonucleotide primer. Proc Natl Acad Sci U S A 85(23),
8998-9002.

Gardner, H., Kerry, K., Riddle, M., Brouwer, S. and
20 Gleeson, L. (1997) Poultry virus infection in Antarctic
penguins [letter]. Nature 387(6630), 245.

Heine, H.G., Haritou, M., Failla, P., Fahey, K. and Azad,
A. (1991) Sequence analysis and expression of the host-
protective immunogen VP2 of a variant strain of
25 infectious bursal disease virus which can circumvent
vaccination with standard type I strains. Journal of
General Virology 72(8), 1835-1843.

Ismail, Y.M., Saif, Y.M. and Moorhead, P.D. (1988) Lack
of pathogenicity of five serotype 2 infectious bursal
30 disease viruses in chickens. Avian Diseases 32(4), 757-
759.

Jackwood, D.J., Saif, Y.M. and Hughes, J.H. (1982)
Characteristics and serologic studies of two serotypes of
infectious bursal disease virus in turkeys. Avian Dis
35 26(4), 871-82.

Kibenge, F.S., Qian, B., Cleghorn, J.R. and Martin, C.K.
(1997) Infectious bursal disease virus polyprotein

in the Netherlands with more virulent vaccines. Summit on Infectious Bursal Disease, 29-32.

5   Lasher, H.N. and Shane, S.M. (1994) Infectious bursal disease. World's Poultry Science Journal 50(2), 133-166.
Lim, B.L., Cao, Y., Yu, T. and Mo, C.W. (1999) Adaptation of very virulent infectious bursal disease virus to chicken embryonic fibroblasts by site-directed

10   mutagenesis of residues 279 and 284 of viral coat protein VP2. J Virol 73(4), 2854-62.
McFerran, J.B., McNulty, M.S., McKillop, T.J., McCracken, R.M., Collins, D.S. and Allan, G.M. (1980) Isolation and serological studies with infectious bursal diesease

15   viruses from fowl, turkey and ducks: demonstation of a second serotype. Avain Pathology 9, 395-404.
Mertens, P.P.C., Jamieson, P.B. and Dobos, P. (1982) In vitro RNA synshesis by infectious pancreatic necrosis virus-associated RNA polymerase. J. Gen. Virol. 59, 47-

20   56.
Muller, H. (1991) Effect of viral structure and replication characteristics on the pathogenesis of infectious bursal disease. Berl. Munch. Tierartzl. Wochenschrift 104(4), 113-117.

25   Mundt, E. and Muller, H. (1995) Complete nucleotide sequences of 5'- and 3'-noncoding regions of both genome segments of different strains of infectious bursal disease virus. Virology 209(1), 10-8.
Mundt, E. and Vakharia, V.N. (1996) Synthetic transcripts

30   of double-stranded Birnavirus genome are infectious. PNAS 93, 11131-11136.
Pattnaik, A.K., Ball, L.A., LeGrone, A.W. and Wertz, G.W. (1992) Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell 69(6), 1011-20.

35   Petek, M., D'Aprile, P.N. and Cancellotti, F. (1973) Biological and phyco-chemical properties of the infectious bursal disease virus (IBDV). Avian Pathology

419-423.

Snyder, D.B., Lana, D.P., Savage, P.K., Yancey, F.S.,
Mengel, S.A. and Marquardt, W.W. (1988a) Differentiation
of infectious bursal disease viruses directly from
infected tissues with neutralizing monoclonal antibodies:
evidence of a major antigenic shift in recent field
isolates. Avian Diseases 32(3), 535-539.

Snyder, D.B., Lutticken, D., Savage, P.K., Yancey, F.S.,
Van der Marel, P., Mengel, S.A., Russek-Cohen, E. and
Marquardt, W.W. (1988b) Differentiation of infectious
bursal disease virus (IBDV) directly from infected
tisues: isolation and geographic distribution of a novel
antigenic variant of IBDV. Procedings of the 23rd
National Meeting on Pouoltry Health and Condemnations.
Vol. October 20-21, 119-128.

Vakharia, V. and Mundt, E. (1996) A method for generating
Birnavirus from synthetic RNA transcripts.

Vakharia, V.N., He, J., Ahamed, B. and Snyder, D.b.
(1994) Molecular basis of antigenic variation in
infectious bursal disease virus. Virus Research 31, 265-
273.

Weiland, J.J. and Dreher, T.W. (1989) Infectious TYMV RNA
from cloned cDNA: effects in vitro and in vivo of point
substitutions in the initiation codons of two extensively
overlapping ORFs. Nucleic Acids Res 17(12), 4675-87.

Yamaguchi, T., Ogawa, M., Inoshima, Y., Miyoshi, M.,
Fukushi, H. and Hirai, K. (1996) Identification of
sequence changes responsible for the attenuation of
highly virulent infectious bursal disease virus. Virology
223, 219-223.

Higuchi. (1990) Recombinant PCR. In: M.A. Innis, D.H.
Gelfnad, J.J. Sninsky and T.J. White (Eds), PCR
protocols, Acedemic Press, San Diego, pp. 177-183.

Muller, H. (1987) Structural and pathogenic qualities of
two serotypes as well as reassortant of the infectious

Mundt, E., B. Kollner, and D. Kretzschmar. (1997) VP5 of
infectious bursal disease virus is not essential for
viral replication in cell culture. J Virol. 71(7), 5647-
51.

Yao, K., Goodwin, M.A. and Vakharia, V.N. (1998)
Generation of a mutant infectious bursal disease virus
that does not cause bursal lesions. J Virol 72(4), 2647-
54.

Higuchi. (1990) Recombinant PCR. In: M.A. Innis, D.H.
Gelfnad, J.J. Sninsky and T.J. White (Eds), PCR
protocols, Acedemic Press, San Diego, pp. 177-183.

Muller, H. (1987) Structural and pathogenic qualities of
two serotypes as well as reassortant of the infectious
virus bursitis (IBDV), Thesis, Justus Liebig-Universitat
Giessen.

Mundt, E., B. Kollner, and D. Kretzschmar. (1997) VP5 of
infectious bursal disease virus is not essential for
viral replication in cell culture. J Virol. 71(7), 5647-
51.

Yao, K., Goodwin, M.A. and Vakharia, V.N. (1998)
Generation of a mutant infectious bursal disease virus
that does not cause bursal lesions. J Virol 72(4), 2647-
54.

1.   An infectious recombinant Infectious Bursal Disease Virus (rIBDV) essentially incapable of growing in a non-bursa-cell or cell derived thereof.

2.   An infectious rIBDV having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus (vvIBDV).

3.   An rIBDV according to claim 1 having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus (vvIBDV).

4.   An rIBDV according to anyone of claims 1 to 3 essentially incapable of growing in a CEF cell, a VERO cell or a QM5 cell.

5.   An rIBDV according to anyone of claims 1 to 4 wherein the amino acid sequence of protein VP2 comprises no asparagine at amino acid position 279.

6.   An rIBDV according to claim 5 wherein the amino acid sequence of protein VP2 comprises aspartic acid at amino acid position 279

7.   An rIBDV according to anyone of claims 1 to 6 wherein the amino acid sequence of protein VP2 comprises no threonine at amino acid position 284.

8.   An rIBDV according to claim 7 wherein the amino acid sequence of protein VP2 comprises alanine at amino acid position 284.

9.   An rIBDV according to claim 8 wherein the amino acid sequence of protein VP2 at least comprises a stretch of amino acids from about position 279 to 289, preferably from about position 229 to 314, most preferably from about position 214 to 328 as found in a vvIBDV isolate such as shown in Table 8.

10. A method for obtaining an infectious recombinant Infectious Bursal Disease Virus (rIBDV) essentially incapable of growing on a non-bursa-cell derived cell comprising transfecting at least one first cell with a

recovered rIBDV in at least one second cell which is permissive for said rIBDV.

5   11.   A method for obtaining an infectious recombinant Infectious Bursal Disease Virus (rIBDV) having retained at least part of the very virulent characteristics of a very virulent Infectious Bursal Disease Virus (vvIBDV) comprising transfecting at least one first cell with a

10   nucleic acid comprising a IBDV genome at least partly derived from a vvIBDV, incubating said first cell in a culture medium, recovering rIBDV from said transfected first cell or said culture medium and propagating said recovered rIBDV in at least one second cell which is

15   permissive for said vvIBDV.

       12.   A method according to claim 11 or 12 wherein said first cell is a non-bursa-cell derived cell.

       13.   A method according to anyone of claims 10 to 12 wherein said second cell is a Bursa-cell derived cell.

20   14.   A method according to anyone of claims 10 to 13 wherein said first cell, such as a CEF cell, a VERO cell or a QM5 cell, is non-permissive for vvIBDV.

       15.   A method according to anyone of claims 10 to 14 wherein said first cell has additionally been provided

25   with a helpervirus or a viral protein derived thereof.

       16.   A method according to claim 15 wherein said viral protein comprises T7-polymerase.

       17.   A method according to anyone of claims 10 to 16 wherein said rIBDV has at least retained the incapacity

30   to substantially be propagated on a vvIBDV non-permissive cell such as a VERO, a QM5 or CEF cell.

       18.   A method according to anyone of claims 10 to 17 wherein said permissive second cell is a primary bursa cell.

35   19.   A method according to anyone of claims 10 to 18 wherein said rIBDV comprises at least a nucleic acid derived from at least a part of genome segment A of

21. A method according to anyone of claims 10 to 20 wherein said rIBDV comprises at least a nucleic acid derived from a serotype II IBDV.

22. A method according to anyone of claims 10 to 21 wherein said rIBDV is lacking at least one immunodominant epitope specific for a serotype I IBDV.

23. An infectious mosaic IBDV (mIBDV) comprising a rIBDV wherein at least one genome segment comprises nucleic acid derived from at least two different Birna virus isolates.

24. A mIBDV according to claim 23 wherein at least one of said isolates is a vvIBDV.

25. A mIBDV according to claim 23 or 24 characterised by its incapacity to substantially be propagated on a vvIBDV non-permissive cell such as a VERO, a QM5 or CEF cell.

26. A mIBDV according to anyone of claims 23 to 25 characterised by its capacity to substantially be propagated on a vvIBDV permissive cell such as a primary bursa cell.

27. A mIBDV according to anyone of claims 23 to 26 wherein at least one of said isolates is a serotype II IBDV.

28. A mIBDV according to anyone of claims 23 to 27 lacking at least one immunodominant epitope specific for a serotype I IBDV.

29. A vaccine comprising a rIBDV according to anyone of claims 1 to 9 or 23 to 28.

The invention relates to recombinant Infectious
Bursal Disease Virus (IBDV) and vaccines derived thereof.
The invention provides infectious recombinant Infectious
Bursal Disease Virus (rIBDV) essentially incapable of
growing in a cell that is not derived from a bursa cell,
or an infectious rIBDV having retained at least part of
the very virulent characteristics of a very virulent
Infectious Bursal Disease Virus (vvIBDV).

Figure 1: antibody titer in broilers (actively vaccinated with intermediate vaccine at 3 weeks)

breakthrough
vv ibdv

breakthrough
interm. ibdv

immunity gap

age in days

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TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CCTTCTACAA YGCTATCATT GATGGTYAGT AGAGATCAGA CAAACGATCG CAGCGATGAC RAACCTGCAA GATCA    150
CEF94-A         .......... C......... ......C... .......... .......... .......... A......... .....    150
D6948-A         .......... T......... ......T... .......... .......... .......... G......... .....    150
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       AACCCAACAG ATTGTTCCGT TCATACGGAG CCTTCTGATG CCAACAACCG GACCGGCGTC CATTCCGGAC GACAC    225
CEF94-A         .......... .......... .......... .......... .......... .......... .......... .....    225
D6948-A         .......... .......... .......... .......... .......... .......... .......... .:...    225
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CCTRGAGAAG CACACTCTCA GGTCAGAGAC CTCGACCTAC AATTTGACTG TGGGGGACAC AGGGTCAGGG CTAAT    300
CEF94-A         ...G...... .......... .......... .......... .......... .......... .......... .....    300
D6948-A         ...A...... .......... .......... .......... .......... .......... .......... .....    300
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       TGTCTTTTTC CCTGGWTTCC CTGGCTCAAT TGTGGGTGCT CACTACACAC TGCAGAGCAA TGGGAACTAC AAGTT    375
CEF94-A         .......... .....A.... .......... .......... .......... .......... .......... .....    375
D6948-A         .......... .....T.... .......... .......... .......... .......... .......... .....    375
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CGATCAGATG CTCCTGACTG CCCAGAACCT ACCGGCCAGY TACAACTACT GCAGGCTAGT GAGTCGGAGT CTCAC    450
CEF94-A         .......... .......... .......... ........T. .......... .......... .......... .....    450
D6948-A         .......... .......... .......... ........C. ---------- ---------- ---------- -----    450
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       AGTGAGGTCA AGCACACTYC CTGGTGGCGT TTATGCACTA AAYGGCACCA TAAACGCCGT GACCTTCCAA GGAAG    525
CEF94-A         .......... ........T. .......... .......... ..C....... .......... .......... .....    525
D6948-A         .......... ........C. .......... .......... ..T....... .......... .......... .....    525
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CCTGAGTGAA CTGACAGATG TTAGCTACAA TGGGTTGATG TCTGCAACAG CCAACATCAA CGACAAAATY GGGAA    600
CEF94-A         .......... .......... .......... .......... .......... .......... .........T .....    600
D6948-A         .......... .......... .......... .......... .......... .......... .........C .....    600
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CGTCCTAGTA GGGGAAGGGG TMACCGTCCT CAGCTTACCC ACATCATATG ATCTTGGGTA TGTGAGRCTY GGTGA    675
CEF94-A         .......... .......... .C........ .......... .......... .......... ......G..T .....    675
D6948-A         .......... .......... .A........ .......... .......... .......... ......A..C .....    675
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CCCCATTCCC GCWATAGGGC TYGACCCAAA AATGGTAGCM ACATGTGACA GCAGTGACAG GCCCAGAGTC TACAC    750
CEF94-A         .......... ..A....... .T........ .........C .......... .......... .......... .....    750
D6948-A         .......... ..T....... .C........ .........A .......... .......... .......... .....    750
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       CATAACTGCA GCCGATGATT ACCAATTCTC ATCACAGTAC CAASCAGGTG GGGTAACAAT CACACTGTTC TCAGC    825
CEF94-A         .......... .......... .......... .......... ...C...... .......... .......... .....    825
D6948-A         .......... .......... .......... .......... ...G...... .......... .......... .....    825
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       YAAYATYGAT GCCATCACAA GCCTCAGCRT YGGGGGAGAR CTCGTGTTTC AAACAAGCGT CCAMGGCCTT RTACT    900
CEF94-A         C..C..T... .......... ........G. T........G .......... .......... ...C...... G....    900
D6948-A         T..T..C... .......... ........A. C........A .......... .......... ...A...... A....    900
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus       GGGYGCYACC ATCTACCTYA TAGGCTTTGA TGGGACWGCG GTAATCACCA GRGCTGTGGC CGCARACAAT GGGCT    975
CEF94-A         ...C..C... ........C. .......... ......A... .......... .G........ ....A..... .....    975
D6948-A         ...T..T... ........T. .......... ......T... .......... .A........ ....G..... .....    975
TY89-A          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
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TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CATCAAACTG GAGATAGTGA CCTCCAAAAG TGGTGGTCAG GCRGGGGATC AGATGTCRTG GTCRGCAAGW GGGAG   1125
CEF94-A      .......... .......... .......... .......... ..A....... .......G.. ...G.....A .....   1125
D6948-A      .......... .......... .......... .......... ..G....... .......A.. ...A.....T .....   1125
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CCTAGCAGTG ACGATCCAYG GTGGCAACTA TCCAGGGGCC CTCCGTCCCG TCACRCTAGT RGCCTACGAA AGAGT   1200
CEF94-A      .......... ........T. .......... .......... .......... ....G..... G......... .....   1200
D6948-A      .......... ........C. .......... .......... .......... ....A..... A......... .....   1200
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    GGCAACAGGA TCYGTCGTTA CGGTCGCYGG GGTGAGCAAC TTCGAGCTGA TCCCAAATCC TGAACTAGCA AAGAA   1275
CEF94-A      .......... ..C....... .......T.. .......... .......... .......... .......... .....   1275
D6948-A      .......... ..T....... .......C.. .......... .......... .......... .......... .....   1275
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CCTGGTYACA GAATACGGCC GATTTGACCC AGGAGCCATG AACTACACAA AATTGATACT GAGTGAGAGG GACCG   1350
CEF94-A      ......T... .......... .......... .......... .......... .......... .......... .....   1350
D6948-A      ......C... .......... .......... .......... .......... .......... .......... .....   1350
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    TCTTGGCATC AAGACCGTMT GGCCAACAAG GGAGTACACT GACTTTCGYG ARTACTTCAT GGAGGTGGCC GACCT   1425
CEF94-A      .......... ........C. .......... .......... ........T. .A........ .......... .....   1425
D6948-A      .......... ........A. .......... .......... ........C. .G........ .......... .....   1425
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CAACTCTCCC CTGAAGATTG CAGGAGCATT YGGCTTCAAA GACATAATCC GGGCCMTAAG GAGGATAGCT GTGCC   1500
CEF94-A      .......... .......... .......... C......... .......... .....A.... .......... .....   1500
D6948-A      .......... .......... .......... T......... .......... .....C.... .......... .....   1500
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    GGTGGTCTCY ACAYTGTTCC CACCYGCCGC TCCCCTAGCC CATGCAATTG GGGAAGGTGT AGACTACCTG CTGGG   1575
CEF94-A      ........C. ...T...... ....T..... .......... .......... .......... .......... .....   1575
D6948-A      ........T. ...C...... ....C..... .......... .......... .......... .......... .....   1575
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CGATGAGGCA CAGGCTGCTT CAGGAACTGC TCGAGCCGCG TCAGGAAAAG CAAGAGCTGC CTCAGGCCGC ATAAG   1650
CEF94-A      .......... .......... .......... .......... .......... .......... .......... .....   1650
D6948-A      .......... .......... .......... .......... .......... .......... .......... .....   1650
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    GCAGCTRACT CTCGCCGCCG ACAAGGGGTA CGAGGTAGTC GCGAATCTRT TYCAGGTGCC CCAGAATCCY GTAGT   1725
CEF94-A      ......G... .......... .......... .......... ........A. .C........ .........C .....   1725
D6948-A      ......A... .......... .......... .......... ........G. .T........ .........T .....   1725
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CGACGGGATT CTYGCTTCAC CTGGGRTACT CCGCGGYGCA CACAACCTCG ACTGCGTGTT RAGAGAGGGT GCCAC   1800
CEF94-A      .......... ..T....... .....G.... ......T... .......... .......... A......... .....   1800
D6948-A      .......... ..C....... .....A.... ......C... .......... .......... G......... .....   1800
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    GCTATTCCCT GTGGTYATYA CGACAGTGGA AGAYGCCATG ACACCCAAAG CAYTGAACAG CAAAATGTTT GCTGT   1875
CEF94-A      .......... .....T..T. .......... ...C...... .......... ..T....... .......... .....   1875
D6948-A      .......... .....C..C. .......... ...T...... .......... ..C....... .......... .....   1875
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----


Consensus    CATTGAAGGC GTGCGAGAAG AYCTCCAACC TCCWTCTCAA AGAGGATCCT TCATACGAAC TCTCTCYGGA CAYAG   1950
CEF94-A      .......... .......... .C........ ...T...... .......... .......... ......T... ..C..   1950
D6948-A      .......... .......... .T........ ...A...... .......... .......... ......C... ..T..   1950
TY89-A       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----
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TY89-A        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus     TGTCTGGGAC GACAGCATTA TGCTGTCCAA AGAYCCCATA CCTCCTATTG TGGGAAACAG YGGAAAYCTA GCCAT  2100
CEF94-A       .......... .......... .......... ...T...... .......... .......... T,....T... .....  2100
D6948-A       .......... .......... .......... ...C...... .......... .......... C.....C... .....  2100
TY89-A        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus     AGCTTACATG GATGTGTTTC GACCCAAAGT CCCMATCCAT GTGGCYATGA CGGGAGCCCT CAAYGCYTRT GGCGA  2175
CEF94-A       .......... .......... .......... ...A...... .....T.... .......... ...T..T.G. .....  2175
D6948-A       .......... .......... .......... ...C...... ......C... .......... ...C..C.A. .....  2175
TY89-A        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus     GATTGAGAAM GTRAGCTTTA GAAGCACCAA GCTCGCCACT GCACACCGAC TTGGCCTYAA GTTGGCTGGT CCCGG  2250
CEF94-A       .........A ..A....... .......... .......... .......... .......T.. .......... .....  2250
D6948-A       .........C ..G....... .......... .......... .......... .......C.. .......... .....  2250
TY89-A        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----

Consensus     WGCATTYGAY GTRAACACCG GGYCCAACTG GGCRACGTTY ATCAAACGTT TYCCTCACAA TCCMCGMGAC TGGGA  2325
CEF94-A       A.....C..T ..A....... ..C....... ...A.....C .......... .C........ ...A..C... .....  2325
D6948-A       T.....T..C ..G....... ..T....... ...G.....T .......... .T........ ...A..C... .....  2325
TY89-A        ---------- ---------- ---------- ---------- ------..... .C........ ...C..A... .....   29

Consensus     CAGGYTMCCY TACCTCAACC TWCCMTAYCT YCCACCMAMW GCWGGACGYC AGTWCSAYCT KGCCMTGGCH GCHTC  2400
CEF94-A       ....C.C..C .......... .A..A..C.. T.....C.AT ..A.....C. ...A.C.C.. T...A....T ..A..  2400
D6948-A       ....C.C..T .......... .T..A..C.. T.....C.AT ..A.....C. ...A.G.C.. G...A....C ..T..  2400
TY89-A        ....T.A..C .......... .T..C..T.. C.....A.CA ..T.....T. ...T.C.T.. G...C....A ..C..  104

Consensus     MGAGTTCAAA GAGACCCCMG AACTCGARRR YGCYGTSMGW GCMATGGAMG CWGCWGCMAA CGTSGACCCA YTRTT  2475
CEF94-A       A......... ........C. .......GAG T..C..CA.A ..A.....A. .A..A..C.. ...G...... C.A..  2475
D6948-A       A......... ........C. .......GAG C..C..CA.A ..C.....A. .A..A..C.. ...G...... C.G..  2475
TY89-A        C......... ........A. .......AGA C..T..GC.T ..A.....C. .T..T..A.. ...C...... T.G..  179

Consensus     CCRMTCWGCD CTCMRBGTST TCATGTGGYT GGAAGARAAY GGGATTGTRA CYGAYATGGC YAACTTCGCM CTCAG  2550
CEF94-A       ..AA..T..A ...AGT..G. ........C. ......G..T .........G. .T..C..... C........A .....  2550
D6948-A       ..AA..T..G ...AGC..G. ........C. ......G..T .........G. .T..T..... C........A .....  2550
TY89-A        ..GC..A..T ...CAG..C. ........T. ......A..C .........A. .C..C..... T........C .....  254

Consensus     CGACCCGAAC GCMCAYMGGA TGMRMAATTT YCTHGCAAAY GCWCCMCARG CMGGMAGCAA GTCGCARAGR GCCAA  2625
CEF94-A       .......... ..C..TC... ..CGA..... T..T.....C ..A..A..A. .A..C..... ......A..G .....  2625
D6948-A       .......... ..C..TC... ..CGC..... T..C.....C ..A..A..A. .A..C..... ......A..A .....  2625
TY89-A        .......... ..A..CA... ..AAA..... C..A.....T ..T..C..G. .C..A..... ......G..G .....  329

Consensus     GTAYGGSACR GCWGGCTACG GAGTGGAGGC YMGRGGCCCC ACDCCAGARG ARGCACAGAG GGARAAAGAC ACACG  2700
CEF94-A       ...C..G..A ..A....... .......... TC.G...... ..A.....G. .A........ ...A...... .....  2700
D6948-A       ...C..G..A ..A....... .......... CC.G...... ..T.....G. .A........ ...A...... .....  2700
TY89-A        ...T..C..G ..T....... .......... TA.A...... ..G.....A. .G........ ...G...... .....  404

Consensus     GATCTCMAAG AAGATGGARA CBATGGGCAT CTACTTYGCA ACACCRGAAT GGGTAGCACT CAAYGGGCAC CGRGG  2775
CEF94-A       ......A... .........G. .C........ ......T... .....A.... .......... ...T...... ..A..  2775
D6948-A       ......A... .........G. .T........ ......T... .....A.... .......... ...T...... ..G..  2775
TY89-A        ......C... .........A. .G........ ......C... .....G.... .......... ...C...... ..A..  479

Consensus     SCCAAGCCCC GGCCAGCTVA AGTACTGGCA RAACACAMGA GAAATACCDG AHCCMAACGA GGACTAYCYA GACTA  2850
CEF94-A       G......... ........A. .......... G......C.. .......G. .C..A..... ......T.T. .....  2850
D6948-A       G......... ........G. .......... G......C.. .......T. .T..A..... ......C.T. .....  2850
TY89-A        C......... ........C. .......... A......A.. ........A. .A..C..... ......C.C. .....  554

Consensus     YGTGCAYGCR GAGAAGAGCC GGTTGGCRTC AGAAGAACAR RTCYTAAGGG CAGCYACGTC GATCTACGGG GCTCC  2925
CEF94-A       C.....T..A .......... .......A.. ..........A A..C...... ....T..... .......... .....  2925
D6948-A       C.....T..A .......... .......A.. ..........A A..C...... ....T..... .......... .....  2925
TY89-A        T.....C..G .......... .......G.. ..........G G..T...... ....C..... .......... .....  629
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TY89-A        .........T ..A....... ....C..... .........C ....GG.... .......... ......G... ..T..  704


Consensus     MAACCARGAR CAGATGAARG AYCTGCTCYT GACTGCGATG GAGATGAAGC ATCGCAATCC CAGGCGGGCT CYACC  3075

CEF94-A       A.....A..A ........A. .T......T. .......... .......... .......... .......... .T...  3075
D6948-A       C.....A..A ........A. .T......C. ..........  .........  .......... .......... .C...  3075
TY89-A        A.....G..G ........G. .C......C. .......... .........  .......... .......... .C...  779


Consensus     AAAGCCMAAG CCAAAACCCA ATGCTCCAWC ACAGAGACCC CCTGGWCGGC TGGGCCGCTG GATCAGGRCB GTCTC  3150

CEF94-A       ......C... .......... ........A. .......... .....T.... .......... .......A.C .....  3150
D6948-A       ......C... .......... ........A. .......... .....T.... .......... .......G.T .....  3150
TY89-A        ......A... .......... ........T. .......... .....A.... .......... .......A.G .....  854


Consensus     TGAYGAGGAC YTKGAGTGAG GYWCCTGGGA GTCTCCCGAC ACCACCCGCG CAGGYGTGGA CACCAATTMR KMMHT  3225

CEF94-A       ...T...... C.T....... .TA....... .......... .......... ....T..... ........CG GACT.  3225
D6948-A       ...T...... C.T....... .CT....... .......... .......... ....C..... ........CG GCCA.  3225
TY89-A        ...C...... T.G....... .CT....... .......... .......... ....T..... ........AA TCAC.  929


Consensus     ASWRMATYCS AAATTGGATC CGTTCGCGGG TCCCC                                              3260

CEF94-A       .CAAC..C.C .......... .......... .....                                              3260
D6948-A       .CAAC..C.C .......... .......... .....                                              3260
TY89-A        .GTGA..T.G .......... .......... .....                                              964
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Consensus   ACGTTAGTGG CTCCTCTTCT TGATGATTCT RCCACCATGA GTGACRTTTT CAAYAGTCCA CAGGCGCGAA GCAMG  150
CEF94-B     .......... .......... .......... G......... .....A.... ...C...... .......... ...C.  150
D6948-B     .......... .......... .......... A......... .....G.... ...T...... .......... ...A.  150


Consensus   ATMTCAGCAG CGTTCGGCAT AAAGCCTACW GCTGGACARG AYGTGGAAGA ACTCYTGATC CCTAARGTYT GGGTG  225
CEF94-B     ..C....... .......... ........T. ........A. .C........ ....T..... ....A..T.. .....  225
D6948-B     ..A....... .......... ........A. ........G. .T........ ....C..... .....G..C. .....  225


Consensus   CCACCTGAGG ATCCSYTKGC CAGCCCTAGT CGWCTGGCMA AGTTCCTCAG RGARAACGGC TACAARRTTY TGCAG  300
CEF94-B     .......... ....GC.T.. .......... ..A.....A. .......... A..G...... ....AG..T. .....  300
D6948-B     .......... ....CT.G.. .......... ..T.....C. .......... G..A...... .....GA..C .....  300


Consensus   CCACGGTCTC TRCCYGAGAA TGAGGAGTAT GAGACCGAYC AAATACTCCC WGACYTAGCW TGGATGMGRC AGATA  375
CEF94-B     .......... .G..C..... .......... ........C. .......... A...T....A ......C.A. .....  375
D6948-B     .......... .A..T..... .......... ........T. .......... T...C....T .......A.G. .....  375


Consensus   GARGGRGCTG TTTTAAAACC MACYCTATCT CTCCCYATTG GAGAYCAGGA GTACTTCCCW AARTACTACC CAACA  450
CEF94-B     ..A..G.... .......... C..T...... .....T.... ....T..... .......... A ..G....... .....  450
D6948-B     ..G..A.... .......... A..C...... .....C.... ....C..... .......... T ..A....... .....  450


Consensus   CAYCGCCCKA GCAAGGARAA GCCCAATGCG TACCCGCCMG AYATCGCAYT ACTCAAGCAG ATGATYTACY TGTTT  525
CEF94-B     ..T.....T. .......G.. .......... ........A. .C......C. .......... .....T...C .....  525
D6948-B     ..C.....G. .......A.. .......... ........C. .T......T. .......... .....C...T .....  525


Consensus   CTCCAGGTTC CMGAGGCCAM MGAKRRCCTW AARGATGARG TMACCCTMYT RACCCAAAAC ATWAGRGAYA ARGCC  600
CEF94-B     .......... .A.......A C..GGG...A ..G.....A. .A.....CT. G......... ..A..G..C. .G...  600
D6948-B     .......... .C.......C A..TAA...T ..A.....G. .C....AC. A......... ..T..A..T. .A...  600


Consensus   TAYGGRAGTG GGACCTACAT GGGACARGCM ACYMGACTTG TKGCYATGAA RGAGGTYGCC ACTGGRAGAA ACCCA  675
CEF94-B     ..T..A.... .......... ......A..A ..TC...... .G..C..... G.....C... .....A.... ......  675
D6948-B     ..C..G.... .......... ......G..C ..CA...... .T..T..... A.....T... ......G.... ......  675


Consensus   AACAARGATC CTCTAAAGCT TGGGTACACY TTTGAGAGCA TMGCSCAGCT ACTTGACATC ACWYTACCGG TAGGC  750
CEF94-B     .....G.... .......... ........T. .......... .C..G..... .......... ..AC...... ......  750
D6948-B     .....A.... .......... ........C. .......... .A..C..... .......... ..TT...... ......  750


Consensus   CCACCCGGTG AGGATGACAA GCCCTGGGTR CCACTCACAA GRGTGCCGTC AMGGATGTTG GTWCTGACGG GMGAC  825
CEF94-B     .......... .......... ........G. .......... .A........ .C........ ..A........ .A...  825
D6948-B     .......... .......... ........A. .......... .G........ .A........ ..T....... .C...  825


Consensus   GTAGATGGSG AMTTTGAGGT TGARGAYTAC CTTCCCAAAA TCAACCTCAA GTCATCAAGT GGACTRCCMT ATGTW  900
CEF94-B     ........C. .C........ ...A..T... .......... .......... .......... ......A..A. ....A  900
D6948-B     ........G. .A........ ...G..C... .......... .......... .......... ......G..C. ....T  900


Consensus   GGTCGCACCA AAGGAGARAC WATTGGSGAG ATGATAGCYA TMTCRAACCA GTTTCTYMGA GAGCTATCAR CRCTG  975
CEF94-B     .......... .......G.. A.....C... .........T. .C..A..... .......CA.. .........A .A...  975
D6948-B     .......... .......A.. T.....G... .........C. .A..G..... .......TC.. .........G .G...  975


Consensus   YTGAAGCARG GTGCAGGGAC AAAARGGGTCR AACAAGAAGA AGCTRCTCAG CATGYTAAGT GACTAYTGGT ACTTA  1050
CEF94-B     T.......A. .......... ...G.....A .......... .....A.... ....T..... ......T.... .....  1050
D6948-B     C.......G. .......... ...A.....G .......... .....G.... ....C..... .....C.... .....  1050


Consensus   TCATGYGGGC TTTTGTTTCC MAAGGCTGAR AGGTACGACA AAAGYACATG GCTCACCAAG ACCCGKAACA TATGG  1125
CEF94-B     .....C.... .......... A........A .......... ....T..... .......... ......G.... .....  1125
D6948-B     .....T.... .......... C........G .......... ....C..... .......... .....T.... .....  1125


Consensus   TCAGCTCCAT CMCCAACACA CCTCATGATC TCWATGATMA CCTGGCCCGT GATGTCCAAY AGCCCAAAYA ACGTG  1200
CEF94-B     .......... .C........ .......... ..T.....C. .......... .........C ........T. .....  1200
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Consensus   ATAWTGGCYC CGGAWGAACC CAAGGCYYTW GTATATGCKG ACAACATATA CATTGTYCAC TCMAACACGT GGTAC   1350
CEF94-B     ...T....C. ....A..... ......TC.T ........G. .......... ......C... ..A....... .....   1350
D6948-B     ...A....T. ....T..... ......CT.A ........T. .......... ......T... ..C....... .....   1350


Consensus   TCAATTGACC TAGAGAAGGG TGAGGCAAAC TGCACKCGYC AACACATGCA RGCCGCMATG TACTACATMC TYACC   1425
CEF94-B     .......... .......... .......... .....T..C. .......... A.....A... ........A. .C...   1425
D6948-B     .......... .......... .......... .....G..T. .......... G.....C... ........C. .T...   1425


Consensus   AGAGGRTGGT CMGAYAACGG YGACCCMATG TTCAATCARA CATGGGCCAC CTTTGCSATG AACATTGCCC CWGCT   1500
CEF94-B     .....G.... .A..C..... C.....A... ........A. .......... ......C... .......... .T...   1500
D6948-B     .....A.... .C..T..... T.....C... ........G. .......... ......G... .......... .A...   1500


Consensus   CTAGTKGTGG ACTCATCRTG YCTGATWATG AACCTKCARA TYAAGACMTA TGGTCAAGGC AGYGGGAATG CAGCC   1575
CEF94-B     .....G.... .......G.. C.....A... .....G..A. .T.....C.. .......... ..C....... .....   1575
D6948-B     .....T.... .......A.. T.....T... .....T..G. .C.....A.. .......... ..T....... .....   1575


Consensus   ACSTTCATCA ACAACCAYCT YYTKAGCACS CTWGTGCTWG ACCAGTGGAA CYTGATGARR CARCCYAGWC CAGAC   1650
CEF94-B     ..G....... .......C.. CT.G.....G ..A.....T. .......... .C......GA ..G..C..A. .....   1650
D6948-B     ..C....... .......T.. TC.T.....C ..T.....A. .......... .T......AG ..A..T..T. .....   1650


Consensus   AGCGARGAGT TCAARTCAAT TGARGACAAG CTRGGYATCA ACTTYAAGAT TGAGAGGTCC ATTGATGAYA TYAGG   1725
CEF94-B     .....G.... ....A..... ...G...... ..A..T.... ....T..... .......... .......T.. .C...   1725
D6948-B     .....A.... ....G..... ...A...... ..G..C.... ....C..... .......... .......C. .T...   1725


Consensus   GGCAAGCTSA GACAGCTTGT CCYCCTTGCA CAACCAGGGT ACCTGAGTGG RGGGGTYGAA CCAGARCAAY CCAGC   1800
CEF94-B     ........G. .......... ..T....... .......... .......... G....T... .....A...T .....   1800
D6948-B     ........C. .......... ..C....... .......... .......... A.....C... .....G...C .....   1800


Consensus   CCAACTGTWG AGCTKGACCT ACTMGGRTGG TCWGCWACWT ACAGCAAAGA TCTYGGGATC TATGTGCCGG TGCTT   1875
CEF94-B     ........T. ....T..... ...A..G... ..A..T..A. .......... ...C...... .......... .....   1875
D6948-B     ........A. ....G..... ...C..A... ..T..A..T. .......... ...T...... .......... .....   1875


Consensus   GACAAGGAAC GCYTATTTTG YTCTGCTGCG TATCCCAARG GRGTAGAGAA YAARAGYCTC AARTCCAARG TYGGG   1950
CEF94-B     .......... ..C....... T......... ........G. .A........ C.G..T... ..G.....A. .C...   1950
D6948-B     .......... ..T....... C......... ........A. .G........ T..A..C... ..A.....G. .T...   1950


Consensus   ATCGAGCARG CATACAARGT WGTCAGGTAY GAGGCGTTGA GGTTGGTAGG TGGTTGGAAC TACCCACTCC TGAAC   2025
CEF94-B     ........G. .......G.. A........T .......... .......... .......... .......... .....   2025
D6948-B     ........A. .......A.. T........C .......... .......... .......... .......... .....   2025


Consensus   AAAGCYTGCA AGAAYAAYGC ARGYGCMGCT CGGCGGCATC TGGAGGCCAA GGGGTTCCCR CTCGAYGAGT TCCTM   2100
CEF94-B     .....C.... ....T..C.. .G.C..C... .......... .......... ..........A .....C.... ....A   2100
D6948-B     .....T.... ....C..T.. .A.T..A... .......... .......... .........G .....T.... ....C   2100


Consensus   GCCGAGTGGT CWGAGYTGTC MGAGTTCGGW GARGCYTTCG AAGGCTTCAA YATCAAGCTG ACMGTAACAY CKGAG   2175
CEF94-B     .......... .T...C.... A........T ..G..C.... .......... T......... ..C......T .T...   2175
D6948-B     .......... .A...T.... C........A ..A..T.... .......... C......... ..A......C .G...   2175


Consensus   AGCCTMGCCG AACTKAACAR RCCAGTACCC CCCAARCCYC CAAATGTCAA CAGACCAGTC AACACYGGKG GRCTM   2250
CEF94-B     .....A.... ....G....A G......... .....G..C. .......... .......... .....T..G. .A..C   2250
D6948-B     .....C.... ....T....G A......... .....A..T. .......... .......... .....C..T. .G..A   2250


Consensus   AAGGCAGTCA GCAAYGCCCT CAAGACCGGY CGGTAYAGRA AYGAAGCCGG ACTRAGTGGY CTCGTCCTYC TAGCC   2325
CEF94-B     .......... ....C..... .........T .....C..G. .C........ ...G.....T .........T. .....   2325
D6948-B     .......... ....T..... .........C .....T..A. .T........ ...A.....C .........C. .....   2325


Consensus   ACMGCMMGMA GCCGWCTRCA RGAYGCAGTY AAGGCCAAGG CAGARGCCGA GAAACTCCAC AAGTCYAAGC CMGAY   2400
CEF94-B     ..A..AA.A. ....T..G.. A..T.....T .......... ....A..... .......... .....C.... .A..C   2400
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Consensus   GTCGCYCACT CAGCACTCGT GGAAACAAGC GACGCYCTTG AAGCRGTYCA GTCRACYTCM GTGTACACYC CMAAG   2550

CEF94-B     .....C.... .......... .......... .....C.... ....A..T.. ...G..T..C ........T. .C...   2550
D6948-B     .....T.... .......... .......... .....T.... ....G..C.. ...A..C..A ........C. .A...   2550


Consensus   TACCCAGARG TYAAGAACCC ACAGACCGCC TCCAACCCCG TTGTTGGGCT CCACCTGCCC GCCAAGAGRG CCACC   2625

CEF94-B     ........A. .C........ .......... .......... .......... .......... ........A. .....   2625
D6948-B     ........G. .T........ .......... .......... .......... .......... ........G. .....   2625


Consensus   GGTGTCCAGG CMGCTCTTCT CGGAGCAGGR ACGAGCAGAC CAATGGGGAT GGAGGCYCCA ACACGGTCCA AGAAC   2700

CEF94-B     .......... .C........ .........A .......... .......... ......C... .......... .....   2700
D6948-B     .......... .A........ .........G .......... .......... ......T... .......... .....   2700


Consensus   GCCGTGAAAA TGGCCAAAMG GCGGCAACGC CAAAARGAGA GCCGCCAAYA GCCATGATGG GAACCACTCA AGAAG   2775

CEF94-B     .......... ........C. .......... .....G.... ........C. .......... .......... .....   2775
D6948-B     .......... ........A. .......... .....A.... ........T. .......... .......... .....   2775


Consensus   AGGACACTAA YCCCAGACCC CGTATCCCCG GCCTTCGCCT GCGGGGGCCC CC                         2827

CEF94-B     .......... T......... .......... .......... .......... ..                         2827
D6948-B     .......... C......... .......... .......... .......... ..                         2827
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Consensus    SNGNYKFDQM  LLTAQNDPAS  INICRDVSRS  DIVRSSIDFG  GVIADNGTIN  AVIFQGDSGS  SSDVSINGSA  SSIAN    150

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    150
D6948-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    150
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    INDKIGNVLV  GEGVTVLSLP  TSYDLGYVRL  GDPIPAIGLD  PKMVATCDSS  DRPRVYTITA  ADDYQFSSQY  Q.GGV    225

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .P...    225
D6948-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .A...    225
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    TITLFSANID  AITSLS.GGE  LVFQTSV.GL  .LGATIYLIG  FDGTAVITRA  VAA.NGLT.G  TDNL.PFN.V  IPT.E    300

CEF94-PP     ..........  ......V...  .......H..  V.........  ..........  ...N....T.  ....L...L.  ...N.    300
D6948-PP     ..........  ......I...  .......Q..  I.........  ..........  ...D....A.  ....M...I.  ...S.    300
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    ITQPITSIKL  EIVTSKSGGQ  AGDQMSWSA.  GSLAVTIHGG  NYPGALRPVT  LVAYERVATG  SVVTVAGVSN  FELIP    375

CEF94-PP     ..........  ..........  .........R  ..........  ..........  ..........  ..........  .....    375
D6948-PP     ..........  ..........  .........S  ..........  ..........  ..........  ..........  .....    375
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    NPELAKNLVT  EYGRFDPGAM  NYTKLILSER  DRLGIKTVWP  TREYTDFREY  FMEVADLNSP  LKIAGAFGFK  DIIRA    450

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    450
D6948-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    450
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    .RRIAVPVVS  TLFPPAAPLA  HAIGEGVDYL  LGDEAQAASG  TARAASGKAR  AASGRIRQLT  LAADKGYEVV  ANLFQ    525

CEF94-PP     I.........  ..........  ..........  ..........  ..........  ..........  ..........  .....    525
D6948-PP     L.........  ..........  ..........  ..........  ..........  ..........  ..........  .....    525
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    VPQNPVVDGI  LASPG.LRGA  HNLDCVLREG  ATLFPVVITT  VEDAMTPKAL  NSKMFAVIEG  VREDLQPPSQ  RGSFI    600

CEF94-PP     ..........  .....V....  ..........  ..........  ..........  ..........  ..........  .....    600
D6948-PP     ..........  .....I....  ..........  ..........  ..........  ..........  ..........  .....    600
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    RTLSGHRVYG  YAPDGVLPLE  TGRDYTVVPI  DDVWDDSIML  SKDPIPPIVG  NSGNLAIAYM  DVFRPKVPIH  VAMTG    675

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    675
D6948-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    675
TY89-PP      ----------  ----------  ----------  ----------  ----------  ----------  ----------  -----


Consensus    ALNA.GEIE.  VSFRSTKLAT  AHRLGLKLAG  PGAFDVNTG.  NWATFIKRFP  HNPRDWDRLP  YLNLPYLPPN  AGRQY    750

CEF94-PP     ....C....K  ..........  ..........  .........P  ..........  ..........  ..........  .....    750
D6948-PP     ....Y....N  ..........  ..........  .........S  ..........  ..........  ..........  .....    750
TY89-PP      ----------  ----------  ----------  ----------  --------...  ..........  .........T  ....F     28


Consensus    HLAMAASEFK  ETPELESAVR  AMEAAANVDP  LFQSALSVFM  WLEENGIVTD  MANFALSDPN  AHRMRNFLAN  APQAG    825

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    825
D6948-PP     D.........  ..........  ..........  ..........  ..........  ..........  ..........  .....    825
TY89-PP      ...L......  ......D...  ..D.......  ..R...Q...  ..........  ..........  ....K.....  .....    103


Consensus    SKSQRAKYGT  AGYGVEARGP  TPEEAQREKD  TRISKKMETM  GIYFATPEWV  ALNGHRGPSP  GQLKYWQNTR  EIPDP    900

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    900
D6948-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    900
TY89-PP      ..........  ..........  ..........  ..........  ..........  ..........  ..........  ...E.    178


Consensus    NEDYLDYVHA  EKSRLASEEQ  ILRAATSIYG  APGQAEPPQA  FIDEVAKVYE  INHGRGPNQE  QMKDLLLTAM  EMKHR    975

CEF94-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    975
D6948-PP     ..........  ..........  ..........  ..........  ..........  ..........  ..........  .....    975
TY89-PP      ....P.....  ..........  V.........  ..........  ......R...  ..........  ..........  .....    253


Consensus    NPRRAPPKPK  PKPNAPTQRP  PGRLGRWIRT  VSDEDLE                                            1012

CEF94-PP     .....L....  ..........  ..........  .......                                           1012
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Consensus    DQILPDLAWM RQIEGAVLKP TLSLPIGDQE YFPKYYPTHR PSKEKPNAYP PDIALLKQMI YLFLQVPEA. ..LKD      150

CEF94-VP1    .......... .......... .......... .......... .......... .......... ..........N EG...      150
D6948-VP1    .......... .......... .......... .......... .......... .......... ..........T DN...      150


Consensus    EVTLLTQNIR DKAYGSGTYM GQATRLVAMK EVATGRNPNK DPLKLGYTFE SIAQLLDITL PVGPPGEDDK PWVPL      225

CEF94-VP1    .......... .......... .......... .......... .......... .......... .......... .....      225
D6948-VP1    .......... .......... .......... .......... .......... .......... .......... .....      225


Consensus    TRVPSRMLVL TGDVDG.FEV EDYLPKINLK SSSGLPYVGR TKGETIGEMI AISNQFLREL S.LLKQGAGT KGSNK      300

CEF94-VP1    .......... ......D... .......... .......... .......... .......... .T........ .....      300
D6948-VP1    .......... ......E... .......... .......... .......... .......... .A........ .....      300


Consensus    KKLLSMLSDY WYLSCGLLFP KAERYDKSTW LTKTRNIWSA PSPTHLMISM ITWPVMSNSP NNVLNIEGCP SLYKF      375

CEF94-VP1    .......... .......... .......... .......... .......... .......... .......... .....      375
D6948-VP1    .......... .......... .......... .......... .......... .......... .......... .....      375


Consensus    NPFRGGLNRI VEWI.AP.EP KALVYADNIY IVHSNTWYSI DLEKGEANCT RQHMQAAMYY ILTRGWSDNG DPMFN      450

CEF94-VP1    .......... ....L..E.. .......... .......... .......... .......... .......... .....      450
D6948-VP1    .......... ....M..D.. .......... .......... .......... .......... .......... .....      450


Consensus    QTWATFAMNI APALVVDSSC LIMNLQIKTY GQGSGNAATF INNHLLSTLV LDQWNLM.QP .PDSEEFKSI EDKLG      525

CEF94-VP1    .......... .......... .......... .......... .......... .......R.. R......... .....      525
D6948-VP1    .......... .......... .......... .......... .......... .......K.. S......... .....      525


Consensus    INFKIERSID DIRGKLRQLV .LAQPGYLSG GVEPEQ.SPT VELDLLGWSA TYSKDLGIYV PVLDKERLFC SAAYP      600

CEF94-VP1    .......... .......... L......... ......S... .......... .......... .......... .....      600
D6948-VP1    .......... .......... P......... ......P... .......... .......... .......... .....      600


Consensus    KGVENKSLKS KVGIEQAYKV VRYEALRLVG GWNYPLLNKA CKNNA.AARR HLEAKGFPLD EFLAEWSELS EFGEA      675

CEF94-VP1    .......... .......... .......... .......... .....G.... .......... .......... .....      675
D6948-VP1    .......... .......... .......... .......... .....S.... .......... .......... .....      675


Consensus    FEGFNIKLTV T.ESLAELN. PVPPKPPNVN RPVNTGGLKA VSNALKTGRY RNEAGLSGLV LLATARSRLQ DAVKA      750

CEF94-VP1    .......... .S.......K .......... .......... .......... .......... .......... .....      750
D6948-VP1    .......... .P.......R .......... .......... .......... .......... .......... .....      750


Consensus    KAEAEKLHKS KPDDPDADWF ERSETLSDLL EKADIASKVA HSALVETSDA LEAVQSTSVY TPKYPEVKNP QTASN      825

CEF94-VP1    .......... .......... .......... .......... .......... .......... .......... .....      825
D6948-VP1    .......... .......... .......... .......... .......... .......... .......... .....      825


Consensus    PVVGLHLPAK RATGVQAALU GAGTSRPMGM EAPTRSKNAV KMAKRRQRQK ESRQ..                          881

CEF94-VP1    .......... .......... .......... .......... .......... ....QP                         881
D6948-VP1    .......... .......... .......... .......... .......... ....--                         879
```

```
CEF94-VP5      .......... ...K...... .......... .......... ....G.....      50

Consensus      VRDLDLQFDC GGHRVRANCL FPW.PWLNCG CSLHTAEQWE LQVRSDAPDC     100
D6948-VP5      .......... .......... ...F...... .......... ..........     100
CEF94-VP5      .......... .......... ...I...... .......... ..........     100

Consensus      PEPTGQLQLL QASESESHSE VKHT.WWRLC TK.HHKRRDL PRKPE          145
D6948-VP5      .......... .......... ....P..... ..W....... .....          145
CEF94-VP5      .......... .......... ....S..... ..R....... .....          145
```

Schematic representation of the used plasmids

300 bp

T7 promotor — 5'-UTR — VP5 — Polyprotein — pVP2 — VP4 — VP3 — 3172 (*Kpn*I) — HDR — T7 Terminator — 3'-UTR (6176 bp)

T7 promotor — 5'-UTR — VP5 — Polyprotein — pVP2 — VP4 — VP3 — HDR — T7 Terminator — 3'-UTR (6176 bp)

VP3 — 3'-UTR (3957 bp)

T7 promotor — 5'-UTR — VP1 — HDR — T7 Terminator — 3'-UTR (5742 bp)

T7 promotor — 5'-UTR — VP1 — HDR — T7 Terminator — 3'-UTR (5742 bp)
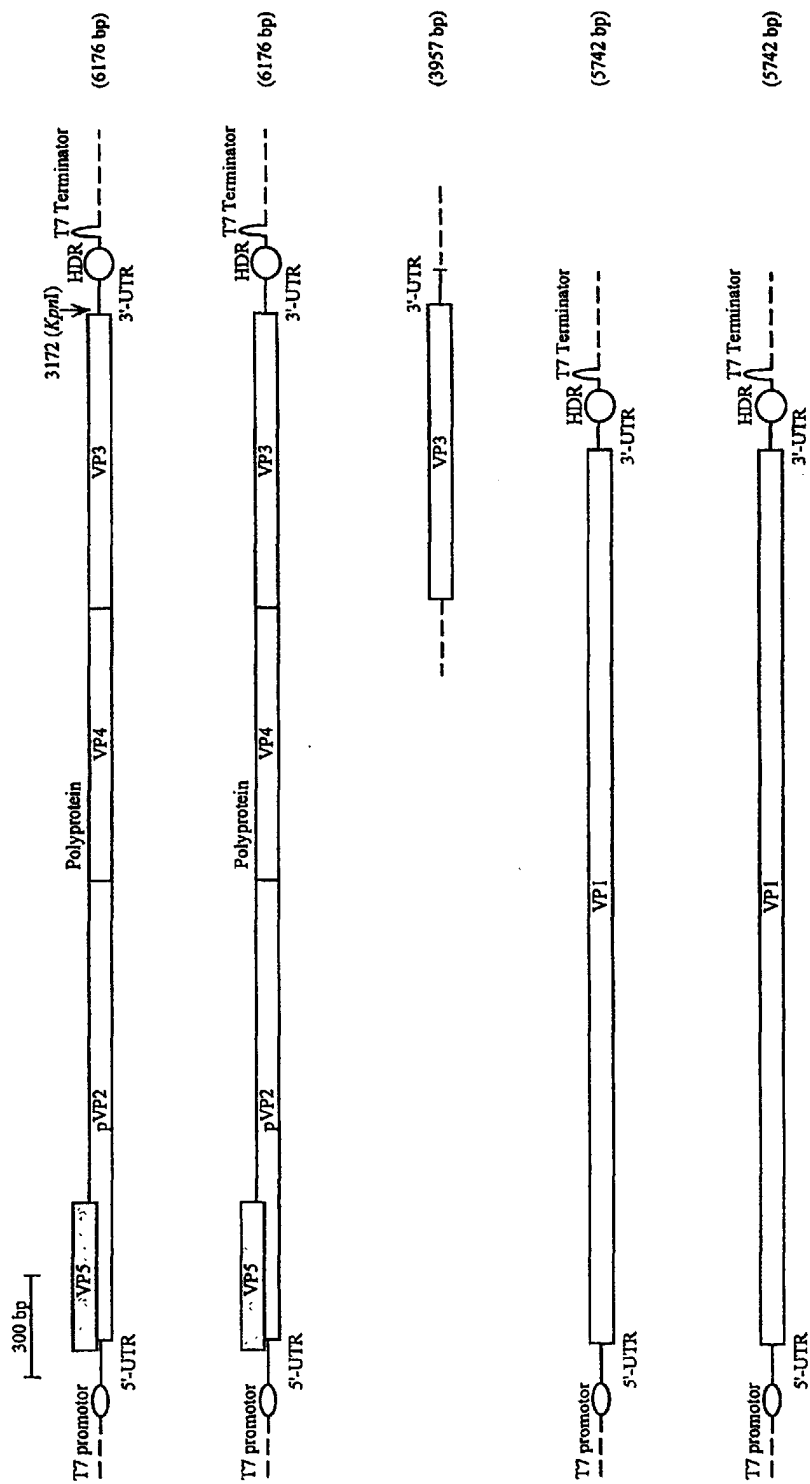
Fig. 5a Schematic representation of the construction of PCR fragment PCR-VP2d
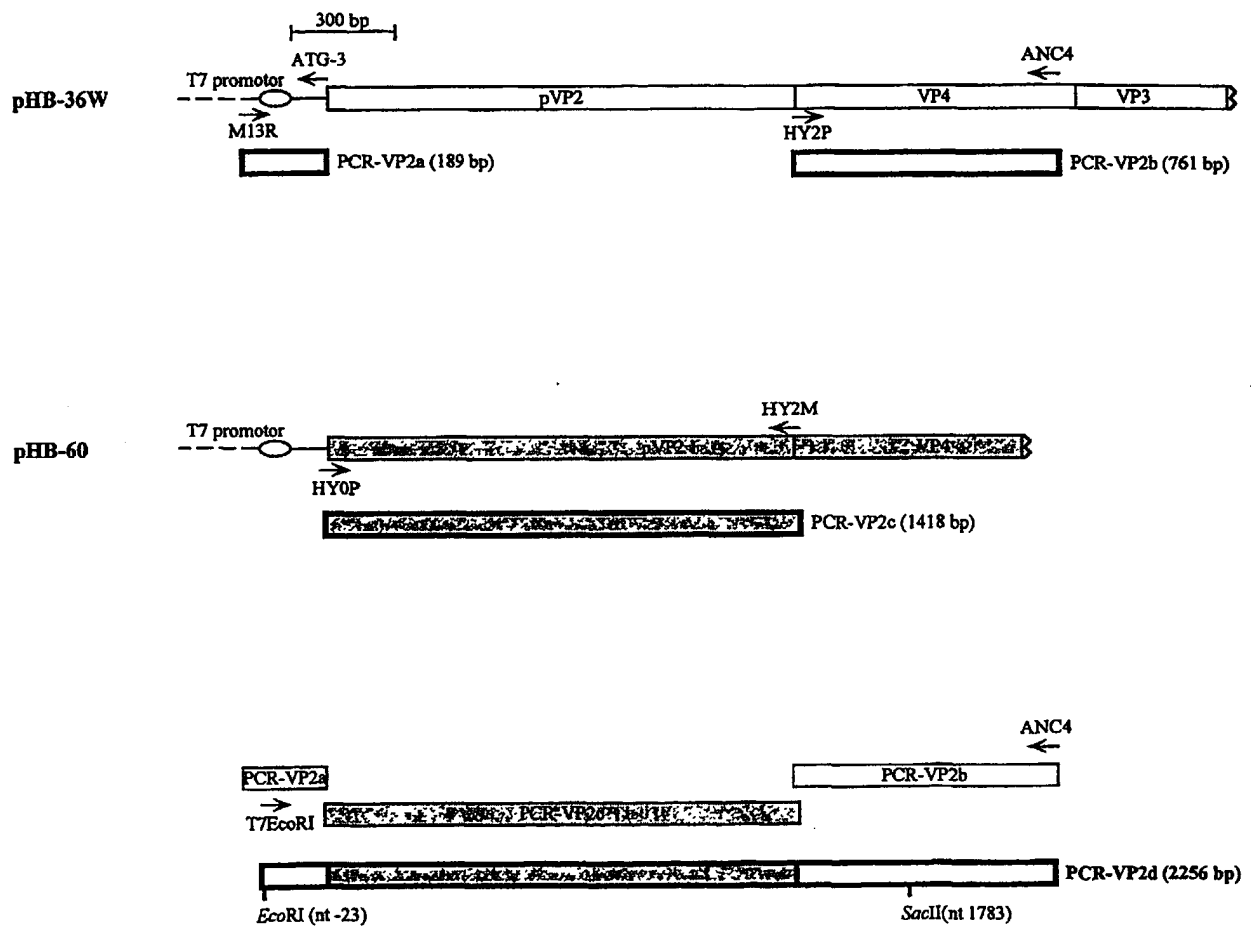
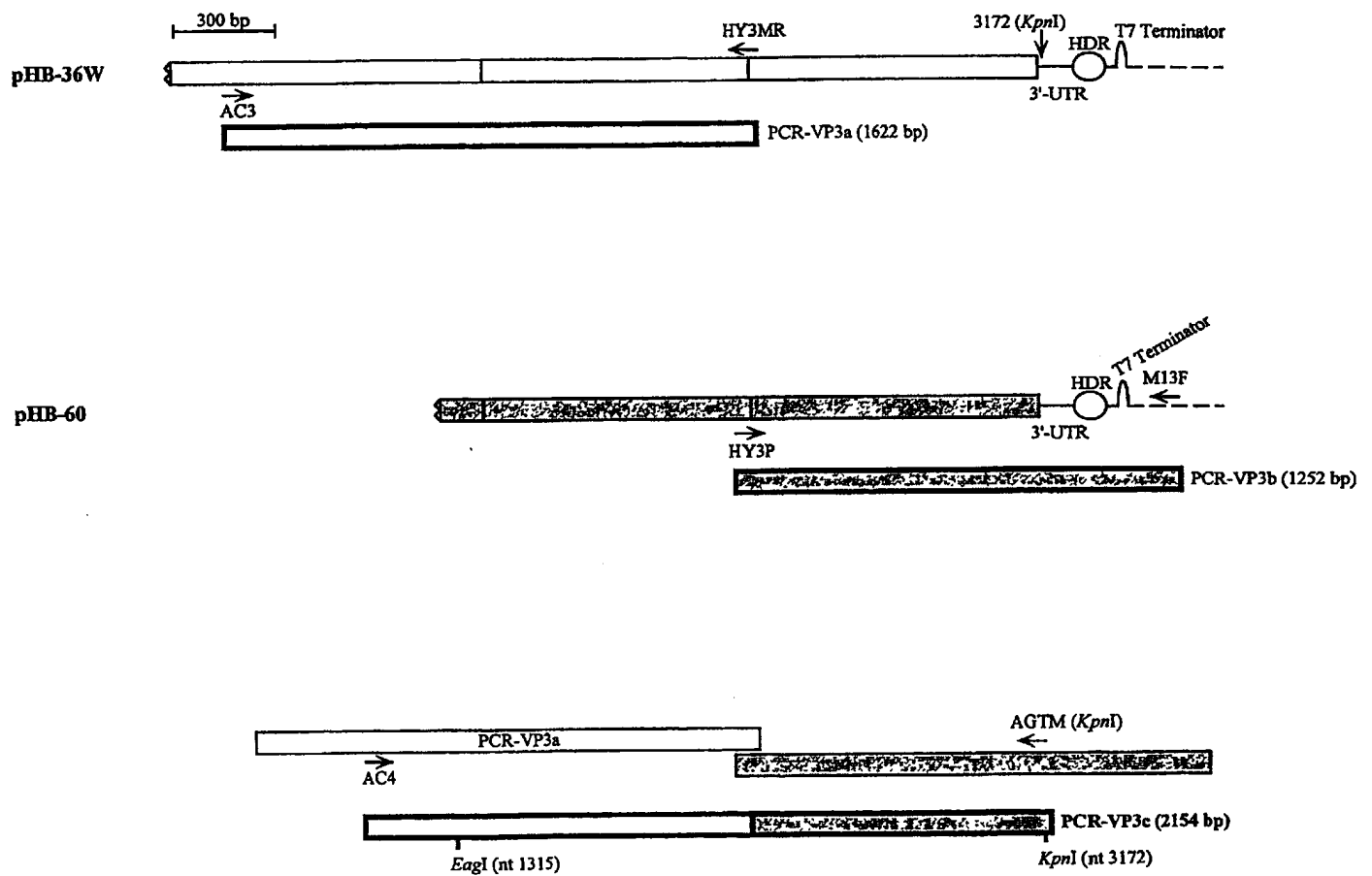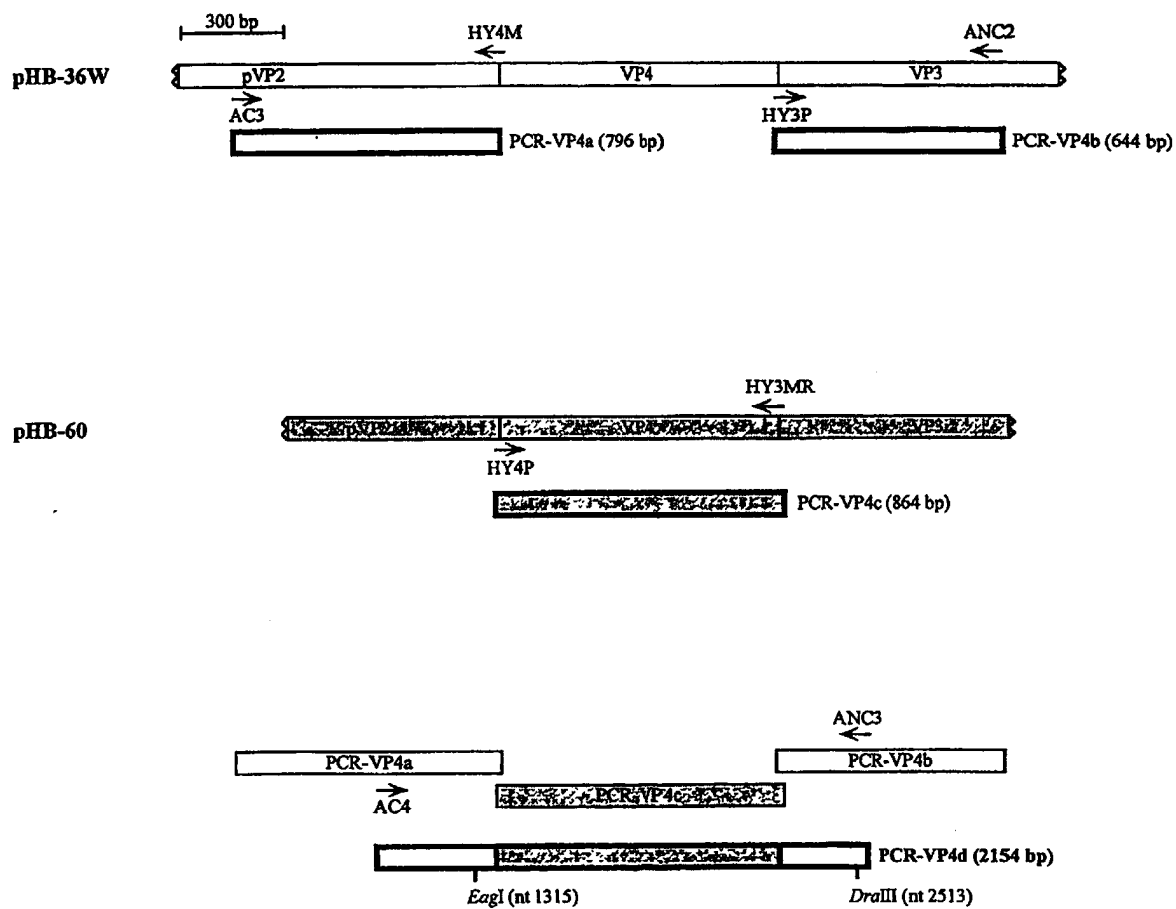# Fig. 5b Schematic representation of the construction of PCR fragment PCR-VP3c

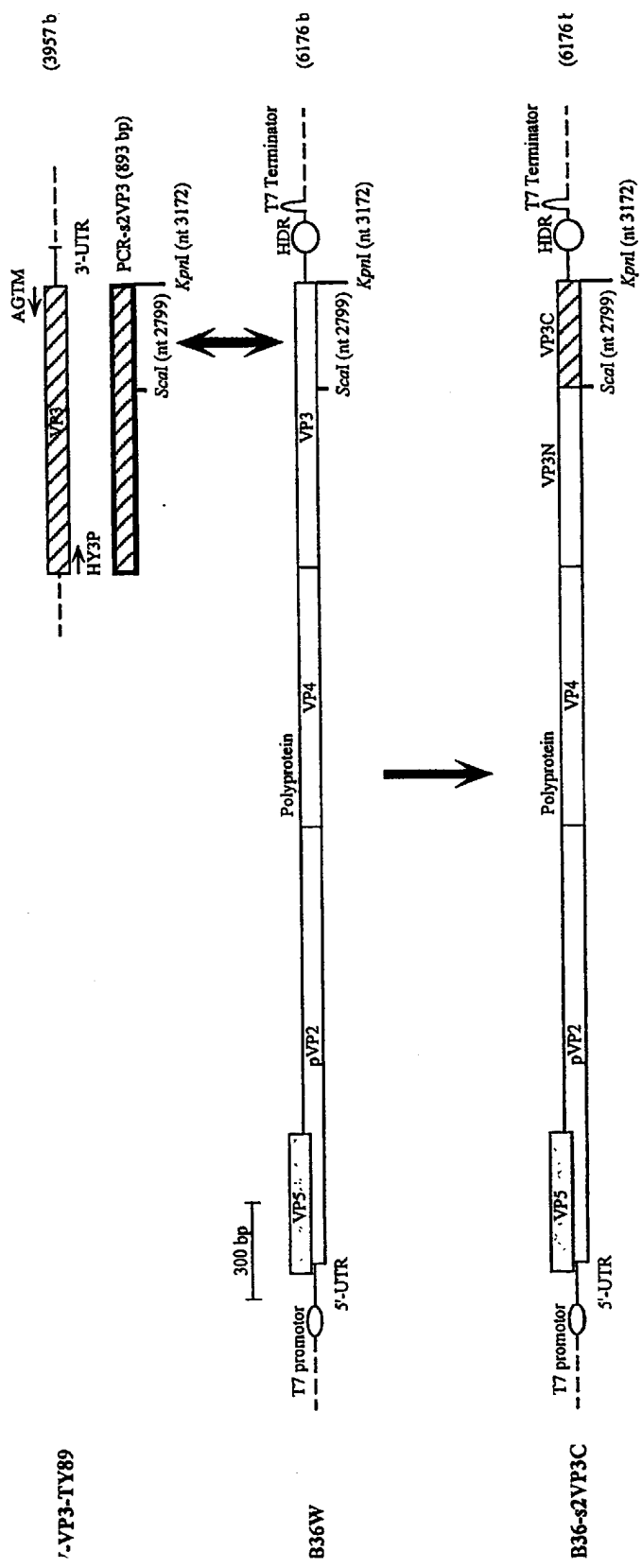**Fig. 5c**   Schematic representation of the construction of PCR fragment PCR-VP4d

Fig. 5d   Schematic representation of the construction of plasmid pHB36-s2VP3

g. 5e Schematic representation of the construction of plasmid pHB36-s2VP3C

'-VP3-TY89
(3957 b

AGTM
VP3
HY3P
3'-UTR
PCR-s2VP3 (893 bp)
ScaI (nt 2799)
KpnI (nt 3172)

B36W

300 bp

T7 promotor
5'-UTR
VP5
pVP2
Polyprotein
VP4
VP3
ScaI (nt 2799)
KpnI (nt 3172)
HDR
T7 Terminator
(6176 b

B36-s2VP3C

T7 promotor
5'-UTR
VP5
pVP2
Polyprotein
VP4
VP3N
VP3C
ScaI (nt 2799)
KpnI (nt 3172)
HDR
T7 Terminator
(6176 t

Fig. 5f

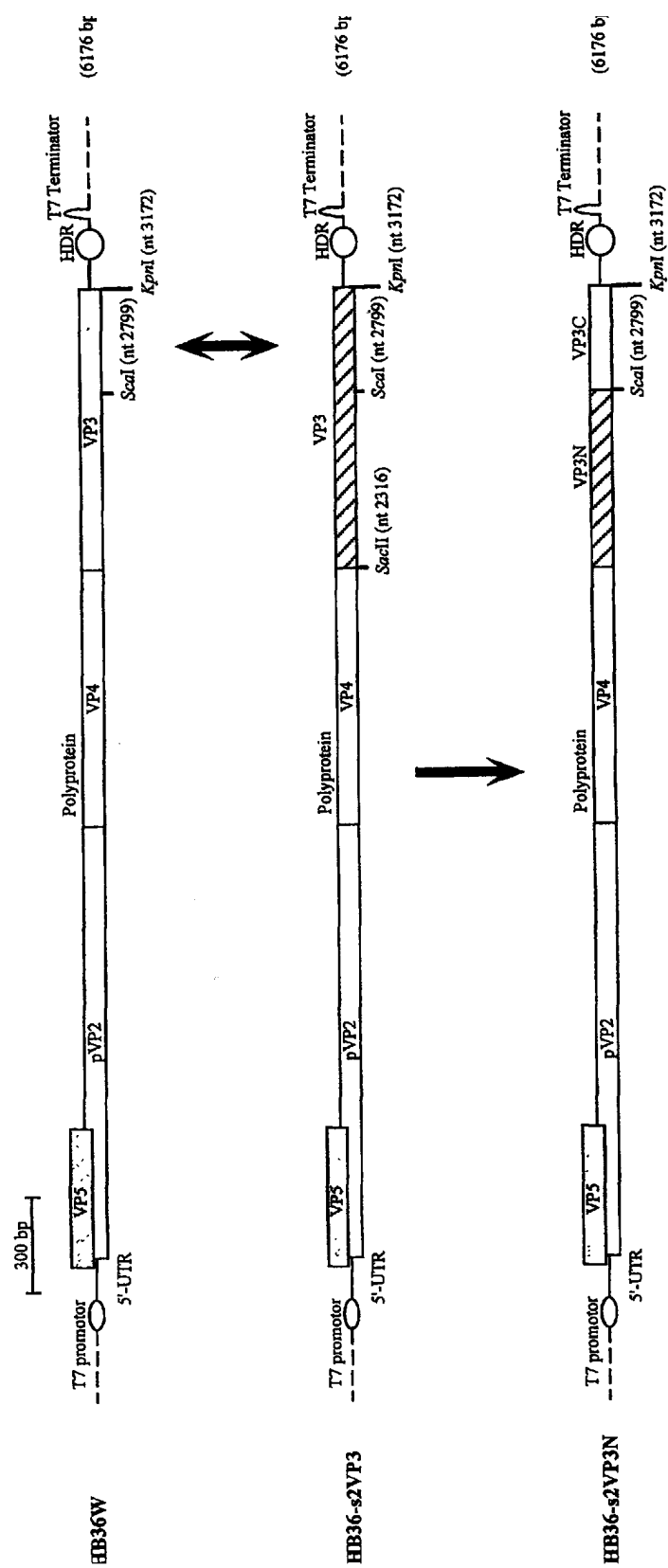## Schematic representation of the construction of plasmid pHB36-s2VP3N

Fig. 5g

Schematic representation of the construction of plasmid pHB60-s2VP3C1